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SELECTIVE TARGETING OF ANTIVIRAL AND
IMMUNOMODULATING AGENTS
IN THE TREATMENT OF ARENAVIRUS INFECTIONS

ANNUAL/FINAL REPORT

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Large multilamellar liposomes were used as a delivery vehicle for the antiviral, ribavirin, and the macrophage activator, muramyl tripeptide (MTP-PE0. The data presented confirms previous observations on the altered tissue (RES) and cellular (macrophage) disposition of intravenously administered drugs and provides evidence to support the therapeutic potential of liposomes in therapy of arenavirus, bunyvirus, orthomyxovirus and herpesvirus infections. Encapsulation of MTP-PE into liposomes enhanced this drug's ability to activate peritoneal, alevolar and liver macrophage antiviral functions. In addition, liposome-encapsulated MTP-PE reduced virus replication in target organs and protected mice from viral infections in which the liver or lung served as the primary site of virus infection. Liposome-encapsulated ribavirin was more effective than free drug in mice infected with influenza virus and in guinea pigs infected with influenza virus and in guinea pigs infected with Pichinde virus. Moreover, the combination of both ribavirin and MTP-PE was superior to either drug alone in the treatment of HSV-1 and Pichinde virus infections. Additional studies have indicated that lipsosomes can provide a vehicle in which both virustatic and immunomodulating agents can be simultaneously delivered to the same call and thus promote drug synergism.

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SUMMARY

An important prerequisite for the therapeutic success with an
antiviral or immunomodulating agent is the ability to reach specific
targets within the host and be maintained at therapeutic levels
without resulting in host toxicity. Drug carries such as liposomes
are useful in this regard since these vehicles can be tailored to
deliver drugs to infected target sites and avoid exposure to
uninfected cells and organs. The data presented in this final report
summarizes our findings using liposomes as carriers for the selective
targeting of antiviral and immunomodulating agents in viral infections
involving the lung, liver, or brain. More specifically, the
therapeutic value of large negatively charged liposomes as vehicles
for the targeted delivery of the broad spectrum antiviral, ribavirin,
and the macrophage activator, muramyl tripeptide (MTP-PE), was
determined in mice or guinea pigs infected with Pichinde, Punta Toro,
influenza, or herpes simplex type 1 viruses.

Our findings have resulted in the following conclusions:

1. Liposomes are a useful vehicle for the targeted delivery of
both antivirals and immunostimulants and have been successfully
employed in the treatment of virus infections in which the lung and
liver are involved.

2. Liposome-encapsulated drugs are retained in the lung, liver
and spleen better than free drugs following intravenous
administration. Both pulmonary, hepatic and splenic macrophages
appear to play significant roles in retention of liposome-encapsulated
drugs.

3. Liposome-encapsulation significantly enhanced the ability of
the synthetic immunostimulant, MTP-PE, to augment macrophage antiviral
functions. Augmentation was observed in macrophages isolated from
diverse anatomical sites (i.e. lung, liver and peritoneum) following
intravenous administration.

4. Because of their predilection for the reticuloendothelial
system, liposomes provide a highly selective means by which
immunostimulants can be delivered to splenic, pulmonary and liver
macrophages, and thereby augment nonspecific immunity to viral
infections in which these organs are the primary sites of virus
replication.

5. Intravenous administration of liposome-encapsulated MTP-PE
was more effective than free drug in enhancing protection to
HSV-1-induced pneumonitis and hepatitis, and to Punta Toro
virus-induced hepatitis. MTP-PE treatment resulted in reduced virus
burdens in target organs and in the production of significant levels
of neutralizing antibody.

6. Intravenous administration of liposome-encapsulated ribavirin was more effective than free ribavirin in the treatment of lethal influenza virus infections. Encapsulation of ribavirin resulted in increased pulmonary retention and a five-fold increase in therapeutic activity.

7. Viral infections of the central nervous system were only partially abrogated with liposome-encapsulated MTP-PE due to the inability of liposomes to penetrate the blood/ brain barrier. In contrast, free MTP-PE, which has been shown to readily cross this barrier more effectively enhanced resistance to herpes encephalitis. The mechanism by which protection occurred is unclear but presumably involves humoral and/or cellular components of CNS immunity.

8. Synergism between ribavirin and MTP-PE was observed in both the Pichinde guinea pig and HSV-1 murine pneumonitis models. This effect was best observed when both drugs were encapsulated in the same vehicle. Thus, liposomes provide a means by which both an immunostimulant and virustatic agent can be simultaneously delivered to the same target site.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the ARMY endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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I. PROBLEM UNDER INVESTIGATION

A number of antiviral compounds such as azidothymidine, phosphonoacetic acid, and dideoxycytidine are quite effective in vitro in limiting virus replication, but because of their toxicity have limited use in vivo. Incorporation of these antivirals into selective targeting vehicles such as liposomes could reduce their toxicity and increase their therapeutic activity by directing drug to infected tissue sites and avoiding contact with uninfected cells.

This study addresses the therapeutic value of liposomes as delivery vehicles for both antivirals and immunomodulators in animals infected with viruses which have a selective tropism for lung, liver, or brain. In this regard, large multilamellar liposomes have been examined for their ability to:

1). Alter the normal tissue distribution and pharmacokinetic characteristics of immunostimulants and/or antivirals following intravenous administration.

2). Deliver immunostimulants and/or antivirals to mononuclear phagocytes at sites of primary virus infection, reduce virus replication, and enhance cellular immunity.

3). Protect antivirals from the normal degradative and clearence mechanisms of the host during the journey from the site of inoculation to the site(s) of infection.

4). Increase uptake and retention of antivirals and/or immunomodulators in infected tissues.

5). Reduce therapeutic dosages normally required by free drug to inhibit virus replication in infected organ and cellular sites.

6). Reduce the toxic effects observed with conventional modes of drug delivery.

7). Provide a vehicle which will allow for the simultaneous delivery of two or more agents and thus promote drug synergism between antivirals and/or immunomodulators.

II. BACKGROUND

Successful drug use in biology and medicine is often prejudiced by the failure of drugs that are otherwise active in vitro to act as efficiently in vivo. This is because in the living animal drugs must, as a rule, bypass or traverse organs, membranes, cells and molecules that stand between the site of administration and the site of action. In practice, however, drugs can be toxic to normal tissues, have limited or no access to the target and be prematurely excreted or inactivated. A number of antiviral agents (e.g., ribavirin, adenine arabinoside, phosphonacetic acid) have been developed which are highly effective in vitro in preventing virus replication and/or cell death; however, their systemic use in man is limited by the induction of toxic effects which occur at dosages required to maintain effective drug concentrations in the infected organ. In particular, sustained treatment often results in leukopenia and subsequent immunosuppression which may affect the outcome of treatment since recovery from most viral infections also involves the cooperation of host immune responses. As in the case of ribavirin where dose limiting toxicity is the development of anemia (1,2), there is now growing optimism that such problems may be resolved with the use of carrier vehicles that will not only protect the nontarget environment from the drugs they carry but also deliver and facilitate their release at the site(s) in which they are needed.

During the past decade, a variety of carrier types have been advocated for the selective targeting of antitumor drugs. Thus, there are numerous reports on the association of drugs such as anthracyclines, methotrexate, bleomycin, chlorambicin, and 1-B-D-arabinofuranosyl cytosine (cytosine arabinoside) with carriers such as DNA (3,4), liposomes (5,6), immunoglobulins (7,8), hormones (9,10), red blood cell ghosts (11) and other proteins (12,13) or polypeptides (14). Most of these carriers have the ability to selectively interact with target cell surfaces and are subsequently endocytosed and transferred to the lysosomal compartment. Free drug is released intracellularly when the bond between the drug and the carrier is hydrolysed by lysosomal enzymes (15). In contrast, liposomes may sometimes deliver their contents directly to the cytoplasm following fusion with the target cell membrane (16). This mode of delivery is useful for drugs which are susceptible to lysosomal enzymes since membrane fusion is a mechanism through which contact with the lysosomal compartment of the cell can be bypassed.

In addition to the examples already cited, liposomes have also been used as carriers for i) the hepatic delivery of arsenicals in the treatment of leishmaniasis (17), ii) iododeoxyuridine in the treatment of herpes keratitis (18), and iii) amphotericin B in the treatment of Candida albicans (19), murine leishmaniasis (20), histoplasmosis (21), and cryptococcosis (22). The mechanism(s) by which liposomes enhanced the chemotherapeutic index of

these drugs has not been defined; nonetheless, increased and prolonged tissue concentration of both iododeoxyuridine and amphotericin B in infected sites are most likely involved. Our rationale for the use of liposomes as carriers in the delivery of antivirals to virus infected tissues or organs in a diseased host is based on previous observations in which these macromolecular carriers provided a therapeutic advantage (23). Ribavirin (1-B-D-ribofulanosyl-2, 4-triazole-3-carboxamide; ICN Pharmaseuticals) was selected as the prototype antiviral to be encapsulated because of its broad spectrum activity and relatively low toxicity . The synthetic analog of muramyl dipeptide, muramyl tripeptide-phosphatidyl ethanolamine (MTP-PE; hereafter designated MTP) manufactured by Ciba Geigy was selected as the immunostimulant of choice because of its lipophilic properties, defined immunoenhancing properties, and potential for use in humans.

III. Results

A. Animal Models

The therapeutic effectiveness of liposome-encapsulated ribavirin and/or MTP-PE were studied in rodent models of the following viral diseases.

Influenza Virus Pneumonitis: The virus used in our laboratory is a mouse adapted H3N2 strain of influenza A virus (Aichi). When 2-10 LD_{50} of this strain is administered intranasally into six to seven week old C3H/HeN mice, death, due to interstitial pneumonia, occurs in five to seven days. Virus is found only in the lungs and mice eventually die of pneumonia. A more detailed description of this model and the effects of immunostimulants on protection have been described by one of us (Gangemi, ref. 24).

HSV-1 Pneumonitis: The virus used in our laboratory is a human isolate (VR3 strain) of type 1 herpes simplex virus (HSV-1) obtained from Dr. Andre Nahmias (Emory University, Atlanta, GA). Intranasal inoculation of three to five week old C3H/HeN mice with 2-10 LD_{50} of virus results in a fulminant pneumonitis and adrenalitis. Death occurs five to eight days following infection. A unique aspect of this model is that encephalitis does not occur. This model was developed by one of us (Dr. M. Nachtigal) and a more detailed description of the histopathological findings has been published (25).

HSV-1 Encephalitis: The virus used to induce encephalitis is a human isolate (MB strain) of type 1 herpes simplex virus obtained from Dr. Richard Whitley (Univ. Ala, Birmingham, AL). Footpad inoculation of four week old C3H/HeN mice results in virus replication in the sciatic nerve, spinal cord and brain. Mice die of encephalitis six to eight days after inoculation. Immunoperoxidase staining for viral antigen has been used to confirm this mode of virus dissemination. This model was developed by us and is routinely used in our laboratory. A continuous source of young C3H/HeN mice is provided by our breeding colony.

HSV-1 Hepatitis: The MB virus strain is used to induce liver disease. When four to five week old C3H/HeN mice are inoculated intravenously with 2-10 LD_{50} of virus, the primary organ of initial infection is the liver. Viremia and dissemination to a number of other organs follows liver infection and death results five to seven days post infection. This model has been described by others (26) and is being used in our laboratory.

Banzi Virus Encephalitis: The seed virus used in our laboratory was obtained from Dr. C.J. Peters (USAMRIID, Fort Detrick, MD). Working stocks of virus are prepared from suckling mouse brains. When

inoculated subcutaneously, this virus replicates in peripheral lymphoid tissue and is carried to the spleen. Viremia results 2-4 days post infection and the virus enters the brain. Encephalitis is observed 6-8 days post infection. Death ensues 8-10 days following the administration of as little as 10 p.f.u.

Punta Toro Hepatitis: The seed virus (Adames strain) used in our laboratory was initially prepared by Dr. D. Pifat (USAMRIID, Fort Detrick, MD). Working stocks of this virus are prepared by passage of cloned virus in Vero cells. Cloned virus was obtained from Dr. W. Sidwell (Univ. Utah) following enrichment and further characterization of the virus prepared by Pifat. Subcutaneous inoculation of 10^4 p.f.u. of our seed virus into 4 week old C3H/HeN mice results in hepatocellular necrosis and death 4-7 days post infection. A unique aspect of this virus infection is the tissue tropism which appears to be restricted to the liver and spleen even in the presence of high levels of circulating virus in the blood. This model of phlebovirus-induced disease was developed by Dr. D. Pifat (Univ. Maryland, Ph.D. dissertation) and is routinely used in our laboratory.

Pichinde Infection of Strain 13 Guinea Pigs: Pichinde virus (ATCC strain AN4763) was obtained from Dr. P. Jarhling (USAMRIID, Fort Detrick, MD) as a 10% spleen suspension. This virus was passed 11 times in guinea pigs prior to receipt. Our stock virus is prepared by inoculating strain 13 guinea pigs subcutaneously with 10^4 p.f.u. At 7 days post infection spleens are removed and the virus plaqued on Vero cells. Clones are picked and this virus passed once again in strain 13 guinea pigs. Subcutaneous inoculation of 250-350g guinea pigs with 10^4 p.f.u. results in death 14-18 days post infection. The virological and pathological findings which we have observed are basically the same as those reported by Jarhling et al (27,28). Replication occurs in the spleen and liver (day 4-7), lung and adrenals (day 5-9), and the spinal cord and brain (day 9-14). While the liver and spleen are heavily infected, much of the pathological damage occurs in the lung. Some inflammation at the hepatic portal triad with antigen deposition is observed; however, the most strikingly consistent finding is interstitial pneumonia with heavy antigen deposition as detected by immunoperoxidase staining.

Pichinde Virus Infection of MHA Hamsters: Pichinde virus (ATCC AN3739) was passed two times in Vero cells to produce stock virus. Subcutaneous inoculation of MHA hamsters with 10^3 p.f.u. of virus results in death 5-7 days after infection. In contrast to strain 13 guinea pigs the liver of MHA hamsters appears to be the primary site of virus replication. Immunoperoxidase staining of liver sections reveals extensive hepatocyte destruction and heavy antigen deposition 4 days following infection. Lung involvement is not as extensive as in the guinea pig.

B. Liposome Preparation and Characterization

Liposomes were prepared by the procedures described by Kende et al. (23) and Fidler et al. (29, 30). The vehicles we selected in the initial phase of our studies were large, negatively charged multilamellar vesicles composed of phosphatidyl choline and phosphatidyl serine at a molar ratio of 7:3, respectively. A small amount of cholesterol (23) was added to ribavirin liposomes to reduce leakage. The source of lipids, preparative procedure, and storage conditions were standardized to avoid possible variation in the efficiency of drug encapsulation or particle size. The size distribution of liposome preparations containing either ribavirin or MTP-PE was determined using flow cytometric procedures. The characteristic light scatter profiles of these vesicles were used to determine the distribution of particle sizes (Figure 1, appendix). Approximately 70% of the liposome encapsulated ribavirin particles produced by our standardized procedures are 1-2 microns, 15% are 2-5 microns, 10% are 5-10 microns, and 5% 10 microns or larger, the later are aggregates. Very similar size distributions appear in MTP-PE liposome preparations. Smaller (80-100 nm) unilamellar liposomes were prepared using a commercially available dialysis instrument (Liposomat). Small unilamellar vesicles have been reported by others to remain in circulation for prolonged periods and to eventually localize in the liver. Unfortunately, these small unilamellar particles were not stable in our hands and encapsulated drugs (e.g. ribavirin) quickly leaked out; thus, their therapeutic value could not be accurately determined.

High performance liquid chromatography procedures were developed to quantitate precise levels of ribavirin per mole of lipid (Figure 2, appendix). Analysis of selected liposome preparations were performed to verify drug dosage and encapsulation efficiency which averaged 15-22%. MTP-PE encapsulation has been determined by others (Ciba Geigy, personal communication) to be greater than 90%. In addition, our liposome preparations were checked for the presence of endotoxin contamination using the Limulus amebocyte gelling assay. Liposome preparations containing detectable endotoxin contamination (test sensitivity = 10 pg) were discarded.

C. MTP-PE Induced Augmentation of Peritoneal, Liver and Alveolar Macrophage Functions

A number of experiments were designed to assess the activation of peritoneal and alveolar macrophages following single or multiple doses of MTP-PE. Figure 3 (appendix) illustrates the inflammatory cell response observed in the peritoneal cavity following a single intraperitoneal (i.p.) inoculation with 100 ug of free or liposome-encapsulated MTP-PE (MTPLIP). Note that the cell response following inoculation with liposome encapsulated MTP-PE preceded that observed with the classical immunostimulant, C. parvum, by several days. Many of the inflammatory cells observed 48 hours post MTP-PE inoculation consisted of macrophages (Figure 4, appendix). The effects of a single or multiple i.p. doses of MTP-PE on inflammatory

responses in the peritoneum are illustrated in Figures 5 and 6
(appendix). Multiple MTP-PE doses were no better than a single dose
in increasing the number of inflammatory cells. Likewise, multiple
i.p. doses did not further enhance or keep the phagocytic activity of
peritoneal macrophages elevated longer than that observed with a
single dose (Figure 7, appendix).

Kinetic analysis of peritoneal macrophage phagocytosis is presented in
Figure 5 (appendix). As illustrated, phagocytosis of radiolabeled
(Cr^{51}) opsonized sheep red blood cells was most active two days after
a single i.p. inoculation with either free or liposome-encapsulated
MTP-PE. Liposome-encapsulated MTP-PE was more effective than free
MTP-PE in augmenting Fc-mediated phagocytic activity of these cells.
Moreover, peak activation occurred several days prior to that observed
with the classical immunostimulant, C. parvum.

The ability of MTP-PE to activate alveolar macrophage functions was
also examined. Mice treated intravenously with 100 ug of MTP-PE were
given intranasal inoculations of 1×10^9 fluorescein-labeled
Staphylococcus aureus organisms. One hour after S. aureus
administration, alveolar macrophages were harvested by transtracheal
lavage and examined by flow cytometry. Figures 8 and 9 (appendix)
illustrate the analytical profiles obtained when the phagocytic
alveolar macrophages containing fluorescent S. aureus organisms are
examined by flow cytometry. Peak movement to the right of the
horizontal scale indicates more vigorous phagocytosis and provides an
indication of the number of phagocytic cells as well as the degree to
which they are phagocytosing S. aureus in vivo. As illustrated in
Figures 8 and 9 (appendix) liposome encapsulated MTP-PE (MTPLIP) was a
more effective alveolar macrophage activator than either sham liposome
(SHLIP) or free MTP-PE. Likewise, the intravenous or intraperitoneal
inoculation of liposome-encapsulated MTP-PE is superior to free MTP-PE
in augmenting the ability of both alveolar and peritoneal macrophages
to kill phagocytosed S. aureus organisms (Figure 10, appendix).

We also examined the enzyme content and antiviral and tumoricidal
activities of peritoneal macrophages recovered from mice several days
following i.p. inoculation of either free or liposome-encapsulated
MTP-PE (Table 1, appendix). Reduction in 5' nucleotidase and alkaline
phosphodiesterase content have been used by Morahan et al. (31) as
markers of macrophage activation. The enzyme reduction observed in
free MTP-PE, liposome-encapsulated MTP-PE and C. parvum treatment
groups correlated with the enhanced antiviral and tumoricidal
functions observed.

Since it is commonly thought that activation of macrophages to the
point in which they display tumoricidal activity is the best measure
of a "fully activated" cell, we examined this function in alveolar
macrophages lavaged from mice receiving intravenous inoculations of
MTP-PE. As illustrated in Table 2 (appendix), liposome-encapsulated
MTP-PE was superior to free MTP-PE in activating alveolar macrophages.

The degree of activation observed with liposome-encapsulated MTP-PE was never as high as that observed with C. parvum but was consistently higher than the activity observed with free MTP-PE.

In addition to our observations on peritoneal and alveolar macrophages, we also examined the effect of MTP-PE treatment on liver macrophages. Since clearance of particles from the blood is a measure of reticuloendothelial function and phagocytosis by liver macrophages, clearance of i.v. administered radiolabeled sheep red blood cells and localization of these cells in the liver was examined. In this assay free MTP-PE was found to be as effective as liposome-encapsulated MTP-PE (Table 3, appendix). This finding was supported by flow cytometric analysis of phagocytic liver cells recovered from mice given fluorescent latex particles 48 hours after treatment. It should be stressed that flow cytometry has been used in this instance to examine the phagocytic activity of individual cells. Liver cells recovered from mice receiving MTP-PE were more highly phagocytic than control cells (Figure 11, appendix).

D. Therapeutic Potential of Liposome-Encapsulated Ribavirin and MTP-PE in the Treatment of Virus-Induced Pneumonitis

We selected large negatively charged multilamellar liposomes as carrier vehicles since previous observations (30) suggested that it was possible to alter tissue distribution and/or persistence of encapsulated drugs. Moreover, it is possible to target drug to infected tissue without exposing nontarget cells or organs. Figures 12 and 13 (appendix) illustrate the tissue distribution of both nonencapsulated and liposome-encapsulated radiolabeled ribavirin following intravenous inoculation. Note that free drug rapidly deposits in the liver and is cleared by 24 hours. In contrast, encapsulated drug accumulates in the liver, spleen and lung and persists in these organs over the twenty-four hour observation period. Moreover, autoradiographic data revealed a selective cellular localization in pulmonary and splenic macrophages. The distribution profile of liposome-encapsulated ribavirin suggests that this method of delivery should be particularly useful in virus infections of the lung and liver.

To examine the efficacy of liposome-encapsulated vs free ribavirin in the treatment of pulmonary infections, mice were given ribavirin several hours after intranasal challenge with influenza or HSV-1. Figure 14 (appendix) illustrates the therapeutic value (prolongation of mean time to death) of ribavirin in either free or encapsulated form. Note that liposome encapsulated ribavirin at 3 mg per mouse per dose was more effective than free drug given at 10 mg per mouse per dose in preventing mortality from influenza infection. Table 4 (appendix) shows that the survivors in the encapsulated ribavirin group all had elevated antiviral antibody titers and were resistant to rechallenge with 100 LD_{50} of virus. In contrast, similar doses of free or liposome-encapsulated ribavirin were not effective in the

HSV-1 model of pneumonitis (Figure 15, appendix) even though this drug was effective in vitro (MED_{50} 30ug).

We also examined the therapeutic activity of free and liposome-encapsulated MTP-PE in the treatment of HSV-1 pneumonitis. When MTP-PE was given several days before, on the day of, and several days after intranasal challenge with virus, encapsulated MTP-PE was more effective than free drug in limiting mortality (Figure 16, appendix). In addition, survivors of MTP-PE treatment had elevated levels of serum neutralizing antibodies and were resistant to rechallenge with 100 LD_{50} of virus (Table 5, appendix). While two or three doses of MTP-PE were effective, a single dose given prophylactically was ineffective (data not shown). Both free and liposome-encapsulated MTP-PE treatments resulted in reduced virus levels in lungs and adrenals of infected mice (Figures 17 and 18, appendix) and no infectious virus was recovered from the blood of treated and infected animals (Table 6).

E. Therapeutic Potential of Liposome-Encapsulated MTP-PE in HSV-1 Hepatitis

Mice, intravenously inoculated with 10^4 p.f.u. of the VR strain of HSV-1, died 6-9 days post infection. Protection (80% survival) was observed when mice were given both intravenous and intranasal therapy with liposome-encapsulated MTP-PE (Figure 19, appendix). In contrast, only 30% survival was observed in mice receiving similar therapy with free MTP-PE. Immunoperoxidase staining of livers from both free and liposome-encapsulated MTP-PE treatment groups revealed less viral antigen and less hepatocyte destruction in those animals receiving liposome-encapsulated MTP-PE. Our rationale for combining both i.v. and i.n. treatment routes was based on our desire to deliver MTP-PE to visceral organs as well as CNS tissue (32).

F. Therapeutic Potential of Liposome-Encapsulated MTP-PE in Punta Toro Virus Infections

Table 7 (appendix) illustrates the augmentation of resistance to Punta Toro virus infection induced by liposome-encapsulated MTP-PE. As shown liposome-encapsulated MTP-PE was effective in protecting mice from death when administered several days prior to and following (D -2,0,2) infection. However, no protection was observed when a single dose of liposome-encapsulated MTP-PE was administered either two days before or on the day of virus challenge. Table 8 (appendix) illustrates the reduction in Punta Toro virus replication following MTP-PE treatment. Note the three to four log reduction in liver and blood virus levels observed in mice receiving encapsulated drug on the days indicated. Survivors had elevated serum neutralizing antibody and were resistant to virus rechallenge thirty days following their initial infection (data not shown).

G. Therapeutic Potential of MTP-PE in HSV-1-Induced Encephalitis

The enhanced effectiveness of liposome-encapsulated MTP-PE observed in HSV-1 hepatitis was similar to the observations reported for the therapy of HSV-1 induced pneumonitis. This finding was not surprising considering the activation of liver macrophages; however, they are in contrast to the protection observed in HSV-1 encephalitis. When 4 week old mice were inoculated with 5×10^5 p.f.u. of the MB strain of HSV-1, death occurred 6-9 days following infection. Significant protection against virus challenge was observed when mice were intranasally inoculated with free MTP-PE on days 0, 1 and 2 post infection (Figure 20 and Table 9, appendix). No enhancement of this effect was observed in mice receiving prophylactic (e.g. day -2) or therapeutic intravenous inoculations of MTP-PE (Figure 21, appendix). The superior protection afforded by free MTP-PE correlated with its ability to suppress virus replication in spinal cords and adrenals of infected animals (Figure 22 and Table 10, appendix). It is interesting that even though free MTP-PE was able to prevent death and inhibit virus replication, latent virus was, in some instances, recovered from CNS tissue (Table 11, appendix). In contrast to the above findings, even free MTP-PE was ineffective in the treatment of Banzi virus induced encephalitis (data not shown).

H.   Therapeutic Activity of MTP-PE in MHA Hamsters Infected with Pichinde Virus

The virological and pathological features observed in MHA hamsters infected intraperitoneally with 5×10^3 p.f.u. of virus were similar to those first described by Buchmeir et al. (33). In this model, the liver is the primary site of virus replication and mice die 8-11 days after infection. We have not been able to show any increase in survival rates following prophylactic or therapeutic MTP-PE treatment but we have some evidence that MTP-PE administered prophylactically may prolong the mean survival times of infected animals (Table 12, appendix).

I.   Therapy of Pichinde Virus Infected Guinea Pigs

Strain 13 guinea pigs infected intraperitoneally or subcutaneously with 1×10^4 p.f.u. of Pichinde virus results in death 15-20 days following infection. Daily intraperitoneal administration of ribavirin (15mg/guinea pig) for 10 days and then every other day to day 19, prolongs the mean survival time and suppresses virus replication at several organ sites; however, most animals die by 30 days post infection (Figure 23 and Tables 13 and 14, appendix). Ribavirin administered intranasally was as effective as i.p. ribavirin therapy. Similarly, liposome-encapsulated MTP-PE administered intranasally was effective in increasing the mean survival time of treated animals.

J.   Combination Therapy

Our previous observations indicated that ribavirin was effective in vitro (MIC$_{50}$ < 30 ug/ml) in suppressing herpesvirus replication (data not shown), whereas in vivo studies (see above) indicated that it was ineffective in our treatment schedule. In addition, we have shown that MTP-PE activates the antiviral functions of macrophages in vitro but a single dose given prophylactically has no effect in vivo in HSV-1 pneumonitis (see above). These observations led us to attempt combination therapy in which both ribavirin and MTP-PE were given in free or liposome-encapsulated form. The results summarized in Figure 24 (appendix) indicate that a single dose of liposome-encapsulated MTP-PE given three days prior to challenge together with liposome-encapsulated ribavirin on the day of challenge was highly effective in preventing death. Note that other combinations given on the day of or several days after virus challenge were ineffective.

Administration of liposome-encapsulated MTP-PE several days prior to ribavirin in mice infected with influenza was more effective than treatment with either ribavirin or MTP-PE alone (data not shown). Moreover, when ribavirin was used in combination with liposome-encapsulated MTP-PE for the treatment of Pichinde virus infections, significant long term protection was observed (Figure 23, appendix). In addition, there was slightly less virus recovered in selected organs obtained from combination treatment groups than from animals receiving either treatment alone (Table 14, appendix).

Initial attempts to load liposomes with both ribavirin and MTP-PE in conventional lipid shells were not successful due to inefficient loading and leakage. More recent attempts to load ribavirin in lyophilized liposomes containing MTP-PE prepared by Dr. Peter Van Hoogevest of CIBA-GEIGY were more successful. As illustrated in Table 15 (appendix), 14% of free C-14 labeled ribavirin was incorporated into liposomes and remained constant over a 24 hour period. Marked protection was observed when MTP-PE liposomes containing ribavirin were administered to mice on the day of and several days following intranasal infection with HSV-1 (Figure 25, appendix). Protection from death (50-80% survival) and survival times of mice which eventually died was significantly better than that observed in mice receiving either free ribavirin or liposome-encapsulated MTP-PE. The above observations suggest that MTP-PE and ribavirin act in either a synergistic or additive fashion and provide a sound rationale for combination therapy in which both antivirals and immunostimulants are simultaneously delivered to a defined site of infection.

IV. Conclusions

The data generated in this three year study is summarized in
Tables 1 and 2 (text pages 23, 24) and has resulted in the following
conclusions:

1. Liposomes are a useful vehicle for the targeted delivery of
    both antivirals and immunostimulants and have been
    successfully employed in the treatment of virus infections in
    which the lung and liver are involved.

2. Liposome-encapsulated drugs are retained in the lung, liver
    and spleen better than free drugs following intravenous
    administration. Both pulmonary, hepatic and splenic
    macrophages appear to play significant roles in retention of
    liposome-encapsulated drugs.

3. Liposome-encapsulation significantly enhanced the ability of
    the synthetic immunostimulant, MTP-PE, to augment macrophage
    antiviral functions. Augmentation was observed in
    macrophages isolated from diverse anatomical sites (i.e.
    lung, liver and peritoneum) following the intravenous
    administration of MTP-PE.

4. Because of their predilection for the reticuloendothelial
    system, liposomes provide a highly selective means by which
    immunostimulants can be delivered to splenic, pulmonary and
    liver macrophages, and thereby augment nonspecific immunity
    to viral infections in which these organs are the primary
    targets of virus replication.

5. Intravenous administration of liposome-encapsulated MTP-PE
    was more effective than free drug in enhancing protection to
    HSV-1-induced pneumonitis and hepatitis, and to Punta Toro
    virus-induced hepatitis. MTP-PE treatment resulted in
    reduced virus burdens in target organs and in the production
    of significant levels of neutralizing antibody.

6. Intravenous administration of liposome-encapsulated ribavirin
    was more effective than free ribavirin in the treatment of
    lethal influenza virus infections. Encapsulation of
    ribavirin resulted in increased pulmonary localization and
    retention and a five-fold increase in therapeutic activity.

7. Viral infections of the central nervous system were only
    partially abrogated with liposome-encapsulated MTP-PE due to
    the inability of liposomes to penetrate the blood/brain
    barrier. In contrast, free MTP-PE, which has been shown to

readily cross this barrier   more effectively enhanced
resistance to herpes encephalitis. The mechanism by which
protection occurred is unclear but presumably involves
humoral and/or cellular components of CNS immunity.

8. Synergism between ribavirin and MTP-PE was observed in both
the Pichinde guinea pig and HSV-1 murine pneumonitis models.
This effect was best observed when both drugs were
encapsulated in the same vehicle. Thus, liposomes provide a
means by which both an immunostimulant and virustatic agent
can be simultaneously delivered to the same target site.

V.   RECOMMENDATIONS

Liposome encapsulation of antivirals and immunomodulators should be pursued in an attempt to enhance the therapeutic index of clinically relevant agents which have limited usefulness due to their toxicity. In this regard, combined chemotherapeutic approaches utilizing both immunomodulators and antivirals in the same liposome need further evaluation. Moreover, since intranasal instillation of liposome-encapsulated ribavirin and MTP-PE was effective in several viral models , additional studies should explore the therapeutic value of aerosol delivery.

TABLE 1   MTP-PE AUGMENTATION OF NON-SPECIFIC IMMUNE FUNCTIONS

Function	Free MTP-PE[*]	Liposome-Encaspsulated MTP-PE[*]	C. Parvum[**]
Macrophage:			
Phagocytosis	++	+++	+++
Bactericidal Activity	++	+++	+++
Antiviral Activity:			
Extrinsic	+	+++	+++
Intrinsic	+	+++	+++
Tumoricidal Activity	+	++	+++
NK Cell Cytotoxicity	−	−	+++
Interferon Induction	−	−	−

[*] 100 ug per mouse
[**] 1.4 mg per mouse

Table 2. Summary of protection data

	Ribavirin		MTP-PE		Combination Therapy
Models	Free Drug	Encapsulated	Free Drug	Encapsulated	Encapsulated MTP + Ribavirin
Pneumonitis Models					
Influenza	+	++++	-	+	++
HSV-1	-	-	+	++++	++++
Hepatitis Models					
HSV-1	-	-	+	++++	ND
Punta Toro	ND	ND	+	++++	ND
Encephalitis Models					
Banzi	ND	ND	-	-	ND
HSV-1	-	-	+++	+	ND
Pichinde Models					
Guinea Pigs	++	++	+/-	+/-	+++
Hamsters	ND	ND	+/-	+/-	ND

- = No protection and no increase in mean survival time
+/- = No protection but increased mean survival time
+ = Minimal protection (<20% survival)
++++ = Significant protection (>80% survival)
ND = Not done

VI. <u>PUBLICATIONS and PRESENTATIONS</u>

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APPENDIX

Appendices

TABLE 1 ENZYME CONTENTS AND ANTIVIRAL AND ANTITUMOR ACTIVITIES OF PERITONEAL MACROPHAGES

TREATMENT GROUP	ENZYME ACTIVITY[a]		ANTIVIRAL ACTIVITY[b] $\log_{10}$ PFU/ml (%reduction)	TUMORICIDAL ACTIVITY[c] (%cytotoxicity)
	5'N	AP		
PBS	27.3	14.7	8.04	22
FREE MTP-PE[d]	18.3	7.6	5.80 (99.42)[f]	41
SHAM LIPOSOME	0.9	18.6	5.36 (99.79)[f]	22
LIPOSOME ENCAPSULATED MTP-PE[d]	1.2	2.2	5.86 (99.34)[f]	40
C. PARVUM[e]	0.2	3.7	4.71 (99.95)[f]	72

a – 5' nucleotidase (5'N) and alkaline phosphodiesterase (AP) activities are presented as nanomoles/min/mg protein. Data provided by Dr. Page Morahan.

b – Virus titers from HSV-1 infected vero cells at macrophage to target cell ratio:1:1. Virus titers determined 72 hours post infection.

c – Isotope release from B-16 target cells at E:T=40:1.

d – 100 micrograms/mouse administered i.p. 2 days prior to harvest.

e – 1.4 mg/mouse administered ip 5 days prior to harvest.

f – Significantly different from PBS control.

TABLE 2 — TUMORICIDAL ACTIVITY OF PULMONARY MACROPHAGES FOLLOWING MTP-PE TREATMENT[a]

TREATMENT GROUP	CELL ASSOCIATED COUNTS (AVERAGE CPM ± STD DEV)	%CYTOTOXICITY[b]
PBS	6498 ± 276	12
SHAM LIPOSOME	6110 ± 473	17
FREE MTP-PE	6112 ± 416	17
LIPOSOME ENCAP-SULATED MTP-PE	5124 ± 735	30[c]
C. PARVUM[d]	3993 ± 263	46[c]
MAXIMUM TARGET CELL COUNTS	7340 ± 461	-

a – 100 μg i.v. 48 hours prior to lung lavage.
b – Effector:target ratio=20:1.
c – P .01 when compared to PBS control group.
d – 1.4 mg i.v. 120 hours prior to lung lavage.

TABLE 3 EFFECT OF MTP-PE TREATMENT ON RETICULOENDOTHELIAL FUNCTION[a]

TREATMENT	CLEARANCE FROM BLOOD (T/2)[b]	LOCALIZATION IN LIVER (CPM per mg. tissue)
PBS	4.3	146
FREE MTP-PE	1.3[c]	217[c]
SHAM-LIPSOME	2.52[c]	186[c]
LIPOSOME ENCAPSULATED MTP-PE	1.81[c,d]	215c
P. ACNES	2.04[c]	178

a – As indicated by clearance of chromium labeled sheep red blood cells from blood and its localization in liver.

b – Minutes taken to clear 50% of chromium labeled sheep red blood cells from circulation. Calculated from the slope of clearance (K value).

c – Significantly different from PBS treated group.

d – Significantly different from SHAM-LIP treated group.

TABLE 4

Antibody Response to Influenza Virus in Ribavirin Survivors*

Mouse #	HI Titer[†]	Virus Challenge[‡]
1	512	R
2	512	R
3	512	R
4	128	R
5	256	R
6	256	R

* - Mice were given intravenous administration of liposome encapsulated ribavirin (3mg/mouse/dose) on the day of intranasal virus challenge (10 LD_{50} of influenza) and two days after infection.

† - Mice were bled on day 21 and the sera assayed for hemagglutination inhibition (HI) antibody.

‡ - Mice were rechallenged two days after bleeding with 100 LD_{50} of influenza virus; R = Resistant.

TABLE 5   Serum Neutralizing Antibody Response
TO HSV-1 Infection in MTP Treated Survivors

| Titer | Number of Animals with Serum-Neutralizing-Titers | |
	FREE MTP	MTP-PE LIP
< 40	3	0
40-100	4	2
100-200	3	3
500-1000	2	8
1000-2500	5	6

Mean Titer: 489   840
$P = 0.09$ by Wilkoxon's Ranking Test, when FREE
MTP-PE and MTP-PE LIP groups were compared.

TABLE 6   VIREMIA[c] AFTER MTP TREATMENT

TREATMENT GROUPS	HOURS POST INFECTION[a]	
	24	48
PBS	—[d]	5
SHAM	—	10
MTP LIP[b]	—	—
FREE MTP[b]	—	—

a – INTRANASAL CHALLENGE WITH 10 LD HSV-1

b – INTRAVENOUS INOCULATION WITH 100μg/MOUSE
    3 DAYS BEFORE AND ON THE DAY OF CHALLENGE

c – PLAQUE FORMING UNITS PER MILLILITER OF BLOOD

d – NO PLAQUES

TABLE 7. Augmentation of Resistance to Punta Toro Virus
Infection by MTP-PE[a]

Treatment[b]	Schedule[c]	%SURVIVAL
Controls	–	0
L-MTP-PE	D-0	20
L-MTP-PE	D-2	10
L-MTP-PE	D-2,0,2	*88

a. Mice were challenged with $10LD_{50}$ of the Adamas strain of Punta Toro.

b. Intravenous administration of liposome-encapsulated MTP-PE, 100μ g/mouse.

c. D = day of treatment.

   *P<.001 when compared to controls.

TABLE 8. Reduction in Punta Toro Virus Replication
in Mice Receiving MTP-PE Treatment

| Group | Virus Titer[a] | |
	Blood	Liver
Control	1.0×10^5	2.0×10^4
L-MTP-PE[b]	$5 \times 10'$	$5 \times 10'$

a. Log_{10} PFU/gram weight.

b. Liposome-encapsulated MTP-PE, 100μ g/mouse i.v. D-2,0,+2 of virus
challenge.

TABLE 9 MEAN SURVIVAL TIMES OF MICE RECEIVING MTP-PE
THERAPY FOR HSV-1 INDUCED ENCEPHALITIS

TREATMENT	TREATMENT SCHEDULE (days)	% SURVIVAL	MEAN SURVIVAL (DAYS)
CONTROL	—	0	8.50
FREE MTP-PE[a]	0, 1, 2	80[b]	13.50[b]
LIPOSOME ENCAP- SULATED MTP-PE[a]	0, 1, 2	50[b]	13.40[b]

a — 100 micrograms MTP-PE administered intravenously on the
indicated days.

b — $p < .05$ when compared to controls.

TABLE 10 INHIBITION OF HSV-1 REPLICATION
FOLLOWING MTP-PE THERAPY

| | Virus Titers | | | |
| | Spinal Cords | | Adrenals | |
Treatment	3 days p.i.	7 days p.i.	3 days p.i.	7 days p.i.
PBS	1.0×10^4	3.7×10^6	2.6×10^5	3.1×10^7
MTP-PE	0	3.8×10^4	5.0×10^3	0

a – 100μg/mouse i.v. on days 0, 1, 2 post-infection.
b – Log_{10} PFU/gram wet weight of tissue. Average titers from two randomly selected mice for each treatment group.

TABLE 11   Rescue of Latent Virus From MTP-PE Treated Survivors

Mouse #	Treatment[a]	Virus Titers[b]					
		7 days post infection			Co-cultivation[c] 31 days post infection		
		Adrenal	Spinal Cord	Brain	Adrenal	Spinal Cord	Brain
1	none	2.0×10^7	3.3×10^6	8.0×10^5	-mouse died-		
2	MTP-PE	0^d	0	0	3×10^2	7.5×10^2	0
3	MPT-PE	0	0	0	0	0	0
4	MPT-PE	0	0	0	0	8×10^2	0

a – MTP-PE (100 μg/mouse) i.v. on the day of and 2 days post footpad infection with 10 LD_{50} of HSV-1.

b – Determined on vero cell monolayers; Log_{10} plaque forming units per gram wet tissue.

c – Minced tissue fragments added to vero cell monolayers and incubated for 10 days. Values represent plaque forming units per ml in culture supernatants.

d – No plaques detected with undiluted samples.

TABLE 12   <u>MTP-PE Therapy of Pichinde Infected MHA Hamsters</u>

<u>Group</u>	<u>Survivors</u>[c]	<u>Mean Survival Time</u>[a]	
		<u>Prophylactic</u>	<u>Therapeutic</u>
PBS	0/10	10.3	10.5
Sham Liposome	0/10	15.2	16.2
Liposome[b] MTP-PE	0/10	15.6	10.4
Free MTP-PE	0/10	21.6	12.0

a. Expressed as days post-infection. Prophylactic treatment includes drug
    2 days prior to, on the day of and '2 days post-infection. Therapeutic
    treatment includes drug on the day of and days 1 and 2 post infection.
b. 150micrograms MTP-PE per dose was administered i.p.
c. Animals were challenged i.p.with 5×10^3 pfu of Pichinde virus.

Table 13 Pichinde Virus Titers in Selected Organs [a]

| | i.p. Treatment Groups: | | | |
	Control	Ribavirin [b]	MTP-PE [c]	Ribavirin+MTP-PE
Liver	1.0×10^7	6.4×10^4	4.0×10^7	3.0×10^5
Lung	9.0×10^8	2.7×10^6	4.0×10^7	8.6×10^6
Spleen	3.0×10^7	2.0×10^6	3.6×10^7	1.7×10^7
Adrenal	2.5×10^8	1.6×10^4	1.0×10^7	4.7×10^5
Spinal Cord	3.6×10^5	< 10	2.0×10^4	< 10
Brain	2.9×10^5	< 10	5.5×10^5	< 10

a. p.f.u./gm.wt.   14 days post-infection
b. 15mg/guinea pig i.p. every day for 10 days then every other day to day 19 post-infection.
c. 200µg/guinea pig i.p. day 0,2,4,6,8 & 10 post-infection.

Table 14 Pichinde Virus Titers in Selected Organs[a]

i.n. Treatment Groups

	Control	Ribavirin[b]	MTP-PE[c]	Ribavirin+MTP-PE
Liver	1.0×10^7	3.3×10^5	2.5×10^6	7.5×10^5
Lung	9.0×10^8	6.3×10^6	3.5×10^7	2.5×10^6
Spleen	3.0×10^7	6.0×10^5	5.5×10^5	3.5×10^5
Adrenal	2.5×10^4	3.5×10^4	5.5×10^6	7.5×10^3
Spinal Cord	3.6×10^4	< 10	7.5×10^3	< 10
Brain	2.9×10^5	< 10	6.0×10^3	< 10

a. p.f.u./gm.wt. 14 days post-infection.
b. 15mg/guinea pig i.n. day 0-10, and day 12,14,16, & 18 post-infection.
c. 200μg/guinea pig i.n. day 0,2,4,6,8 & 10 post-infection.

Table 15   Encapsulation of ribavirin into freeze-dried
liposomes containing MTP-PE

	CPM/0.05ml	%Encapsulation efficiency
C^{14}-RIB/MTP (after swelling)	1,623,300	-
Supernatent 1st wash	747,300	-
Supernatent 2nd wash	164,642	-
Supernatent 3rd wash	(ND)	1
Washed Liposomes (day 1)	220,820	14
Washed Liposomes (day 2)	113,520	7

5ml of Ribavirin (75mg/ml) spiked with 0.1ml of C^{14} RIB (spec act. 56.3m
Ci/mM) was added to lyophilized liposomes containing 1mg of MTP-PE and
allowed to swell at 40°C for 60 minutes. Loaded liposomes were washed 3x
in Dulbeco's PBS prior to use.

FIGURE 1

FLOW CYTOMETRIC ANALYSIS OF THE SIZE
DISTRIBUTION OF SHAM AND MTP LIPOSOMES
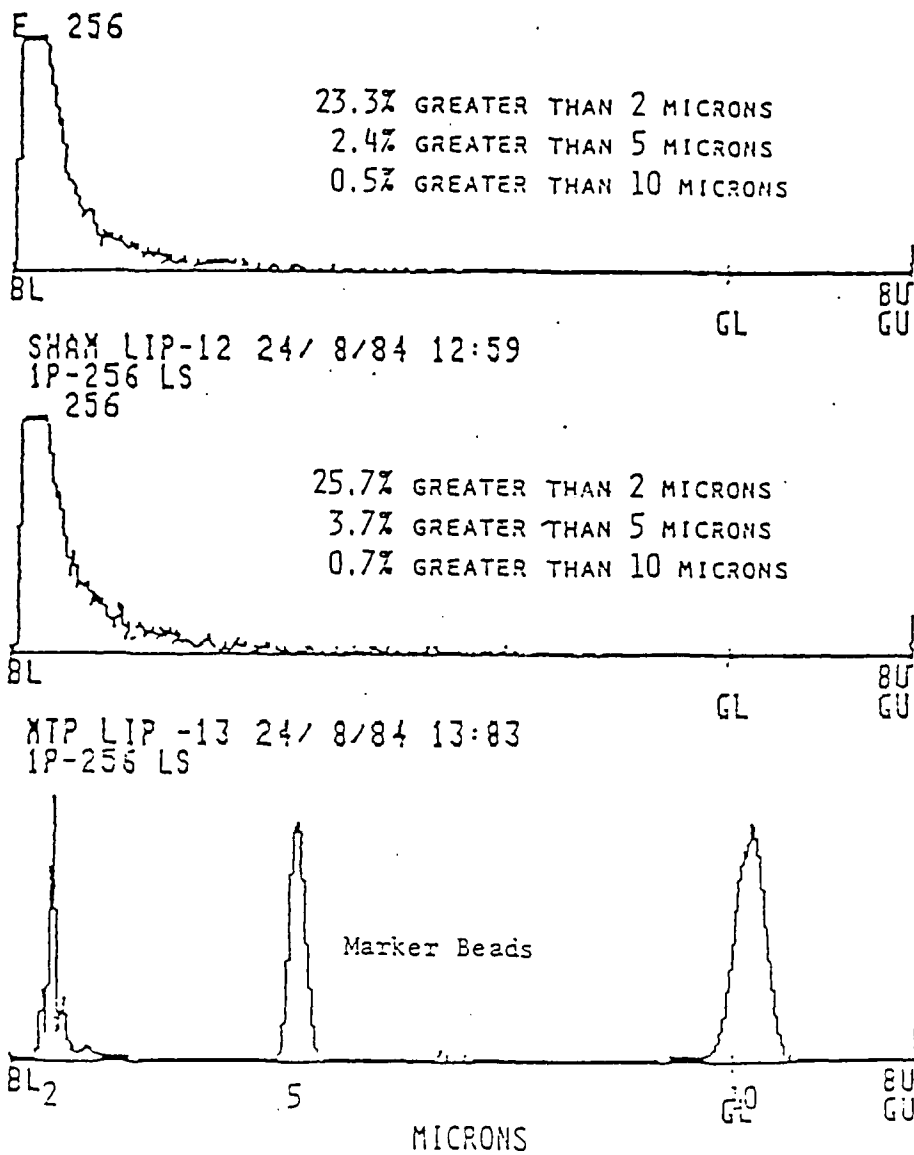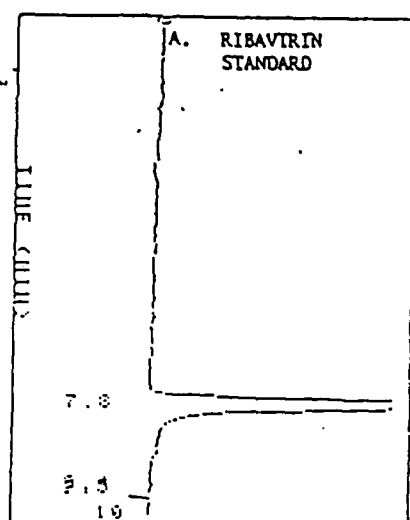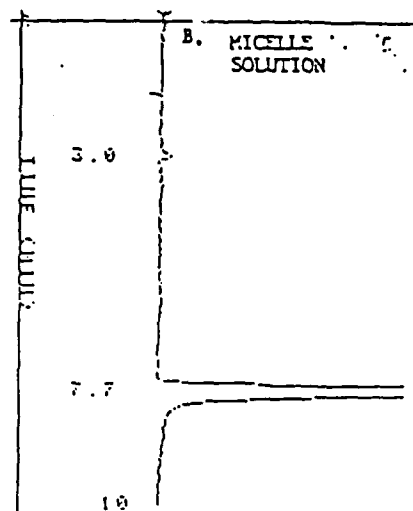
E 256

23.3% GREATER THAN 2 MICRONS
2.4% GREATER THAN 5 MICRONS
0.5% GREATER THAN 10 MICRONS

BL   GL   BU
                                              GU

SHAM LIP-12 24/ 8/84 12:59
1P-256 LS

256

25.7% GREATER THAN 2 MICRONS
3.7% GREATER THAN 5 MICRONS
0.7% GREATER THAN 10 MICRONS

BL   GL   BU
                                              GU

MTP LIP -13 24/ 8/84 13:83
1P-256 LS

Marker Beads

BL 2   .5   GL0   BU
                                       GU

MICRONS
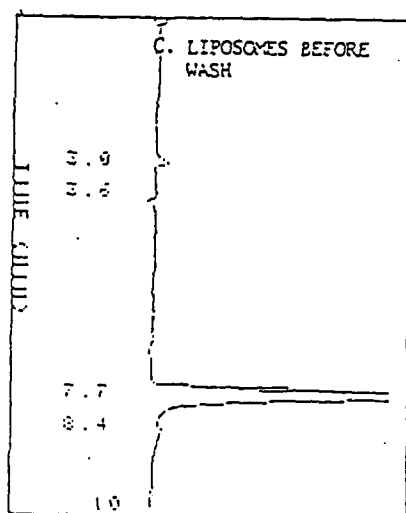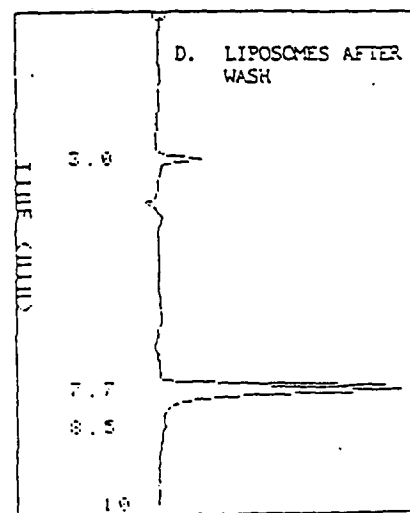
FIGURE 2   49

Figure 2. HPLC analysis of liposome-encapsulated ribavirin Samples were seen in an isocratic made through an ODS-HPLC columns (ultrasphere). The solvent consisted 0.005M sodium acetate, 0.0025M sodium heptane-sulfonate pH 7.0. UV adsorbance at 212 was recorded.

FIGURE 3

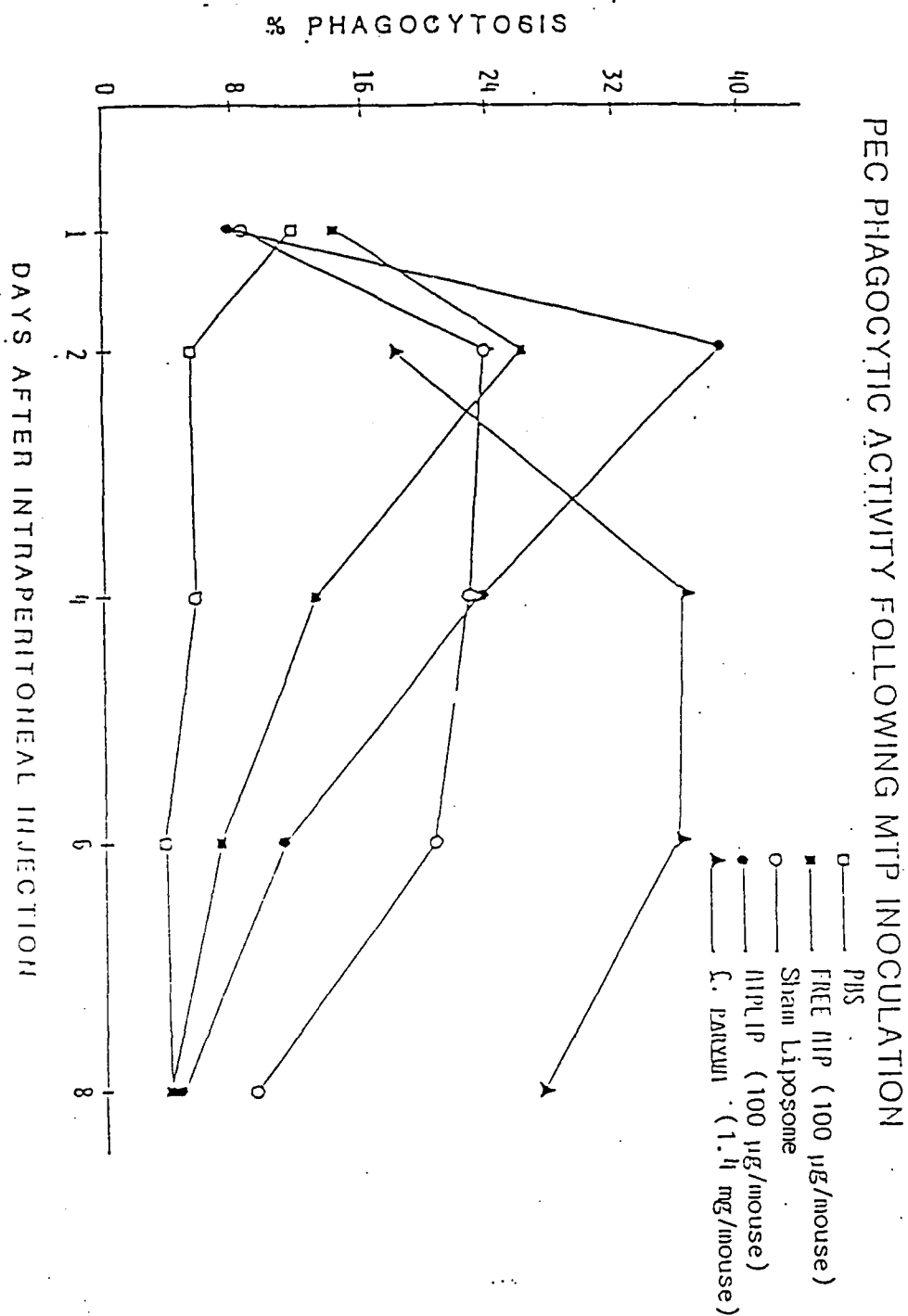CELL COUNT·PER MOUSE (x10^6)

DAYS AFTER INTRAPERITONEAL INJECTION

PERITONEAL LAVAGE CELL COUNTS

○——— PBS
△——— FREE MTP (100 μg/mouse)
✱——— Sham Liposome
◐——— MTPLTP (100 μg/mouse)
▫——— C. parvum (1.4 mg/mouse)

FIGURE 4

INFLAMATORY MACROPHAGE RESPONSE
FOLLOWING MTP INOCULATION

% MACROPHAGE

DAYS POST INOCULATION

△   MTP LIP (100 µg/mouse)
O   FREE MTP (100 µg/mouse)
□   SHAM LIPOSOMES
✱   C. PARVUM (1.4 mg/mouse)

FIGURE 5

% PHAGOCYTOSIS

DAYS AFTER INTRAPERITONEAL INJECTION

PEC PHAGOCYTIC ACTIVITY FOLLOWING MTP INOCULATION

PBS
FREE MTP (100 μg/mouse)
Sham Liposome
MTPLIP (100 μg/mouse)
C. parvum (1.4 mg/mouse)

FIGURE 6

CELL COUNTS PER MOUSE (10^7)

EFFECTS OF MULTIPLE DOSES OF MTP-PE
ON PERITONEAL LAVAGE CELL COUNTS

NUMBER OF MTP-PE DOSES

FREE MTP (100μg/mouse/dose)

MTP LIPOSOMES (100μg/mouse/dose)

* 24 HOURS AFTER IP INJECTION

** 48 HOURS AFTER IP INJECTION

FIGURE 7

EFFECTS OF MULTIPLE DOSES OF MTP-PE ON PHAGOCYTOSIS

FREE MIP   (100µg/mouse/dose)
MIP LIPOSOMES (100µg/mouse/dose)
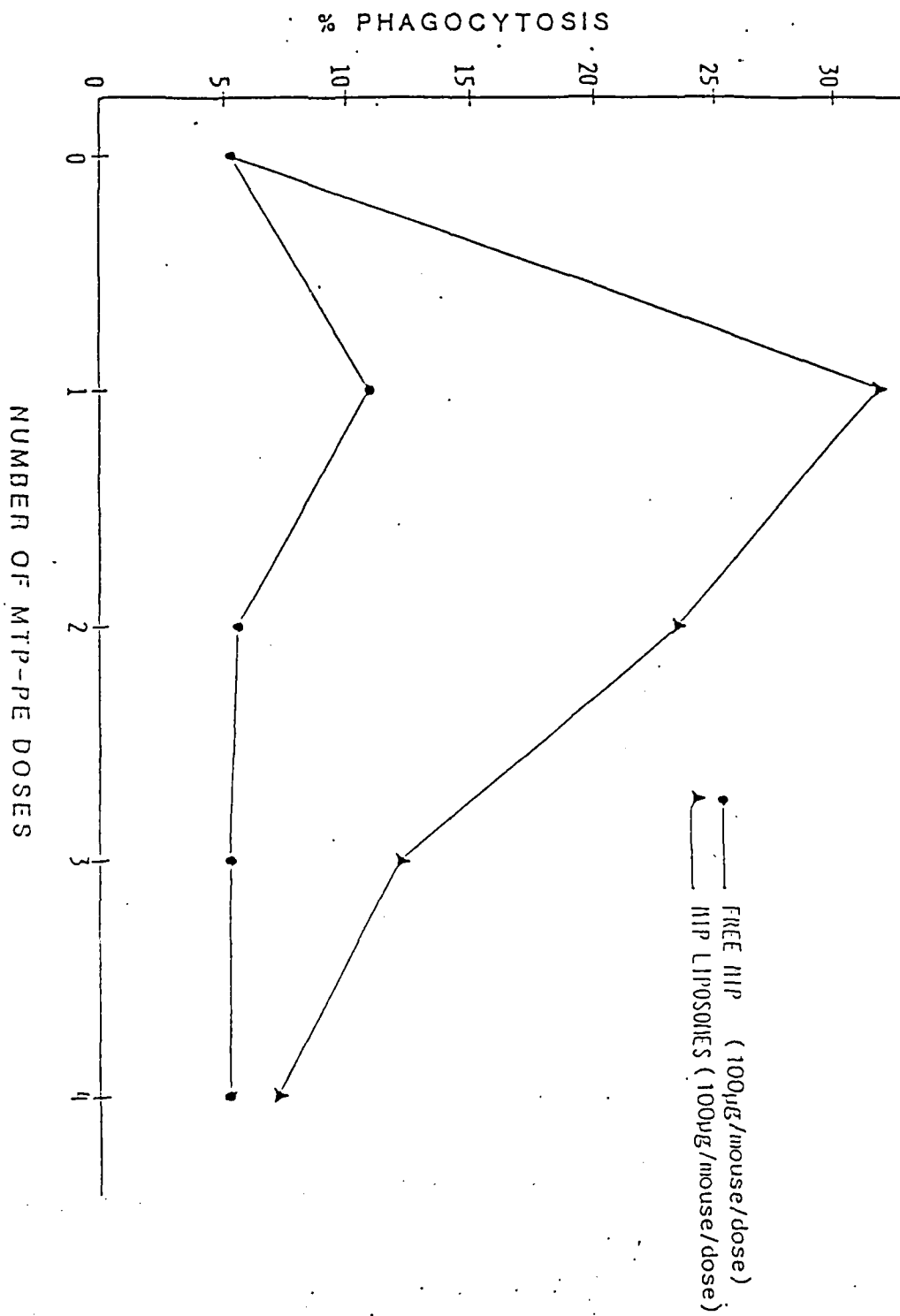
FIGURE 8

CELL SORTER ANALYSIS OF ALVEOLAR MACROPHAGES FOLLOWING PHAGOCYTOSIS OF FLUORESCEIN LABELED S. AUREUS
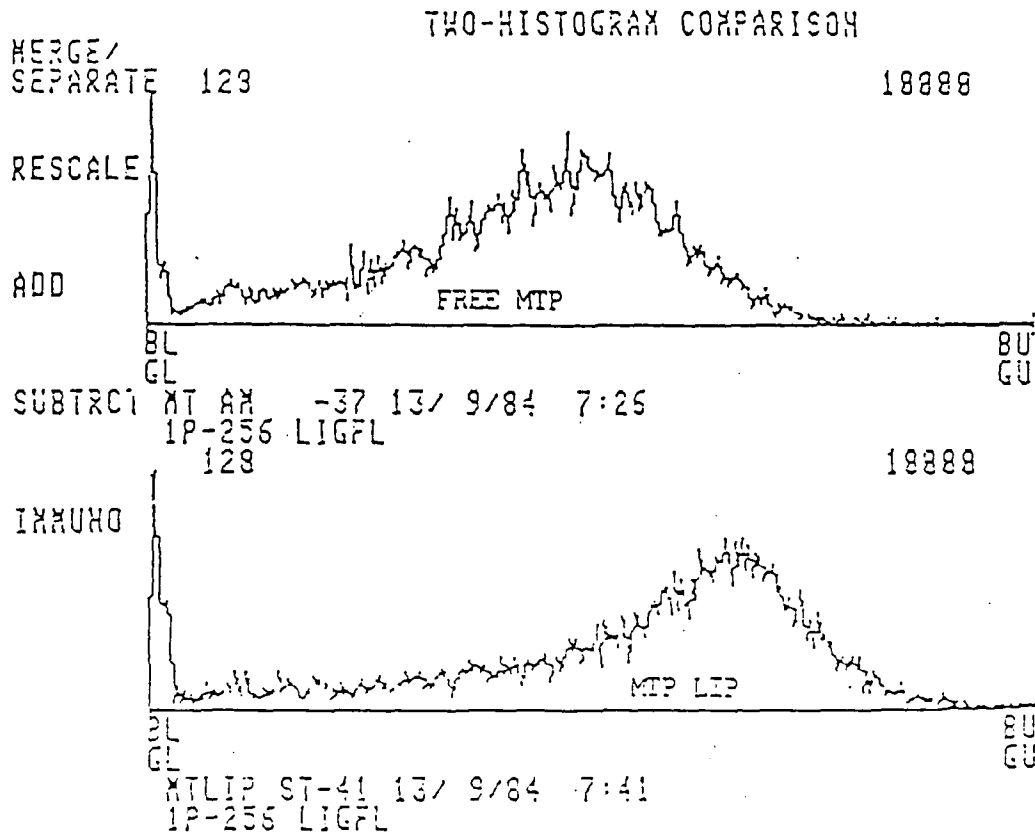
Figure 8 - Phagocytic Activity of Alveolar Macrophages Following MTP Treatment. Three groups of mice were inoculated intravenously with 0.2 ml of either sham liposome, liposome encapsulated MTP (100 ug/mouse), or free MTP (100 ug/mouse) 48 hrs prior to the instillation of 10^6 fluorescein labeled Staphylococcus aureus organisms. Lungs were transtracheally lavaged one hour after S. aureus administration and examined in an Epics IV Flow cytometer. The histograms represent the average effect observed from three mice in each group.

FIGURE 9

CELL SORTER ANALYSIS OF ALVEOLAR MACROPHAGES
FOLLOWING PHAGOCYTOSIS OF FLUORESCEIN LABELED
S. AUREUS

TWO-HISTOGRAM COMPARISON

```
MERGE/
SEPARATE    128                              18668

RESCALE

ADD                          Sham Liposome
            BL                                    BU
            GL                                    GU
SUBTRCT  SHLIP ST-48 13/ 9/84  7:39
         1P-256 LIGFL
            128                              18888

IMMUNO

                                  MTP LIP
            BL                                    BU
            GL                                    GU
         MTLIP ST-41 13/ 9/84  7:41
         1P-256 LIGFL
```
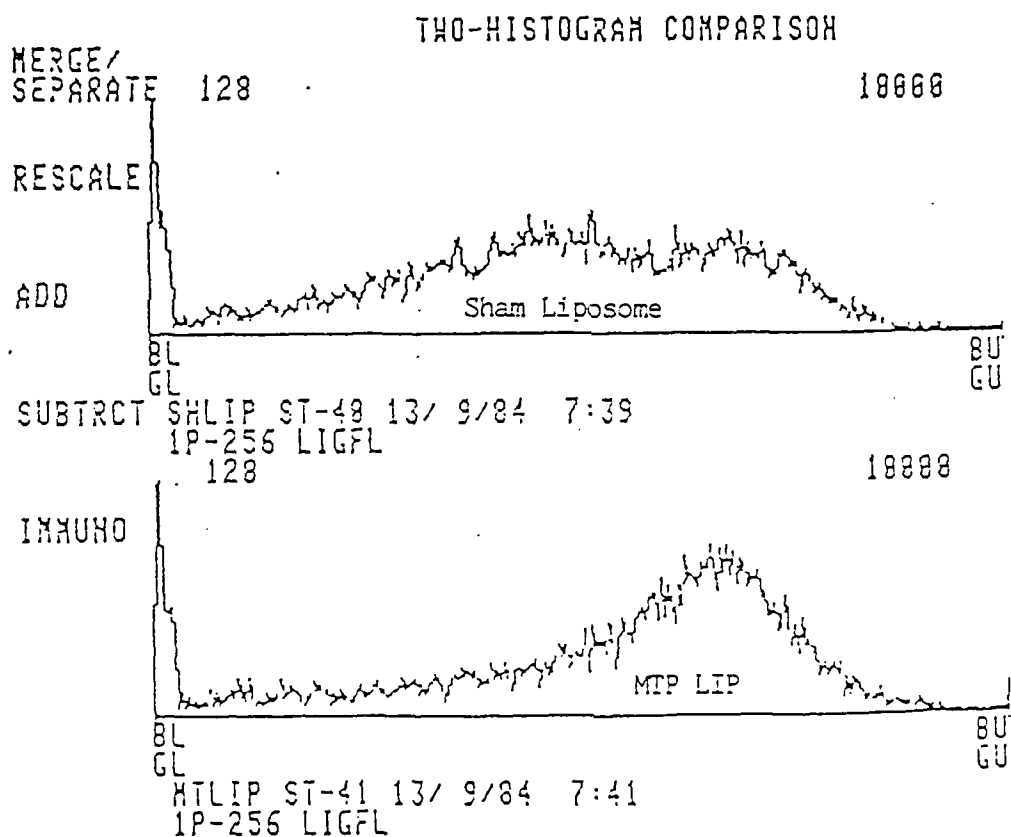
Figure 9 - Phagocytic Activity of Alveolar Macrophages Following
MTP Treatment. See figure 8 legend for details.

FIGURE 10   57

BACTERICIDAL ACTIVITY OF PERITONEAL MACROPHAGES
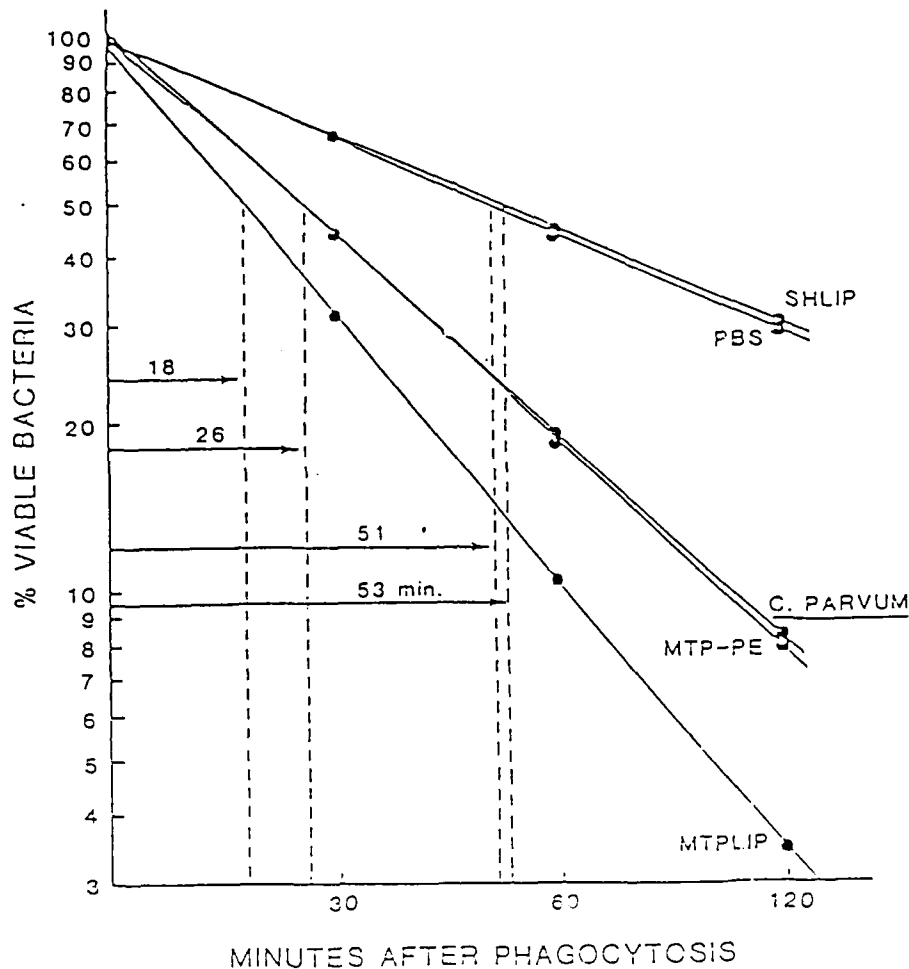
Figure 10 - Microbicidal Activity of Peritoneal Macrophages Following MTP Treatment. Mice were inoculated intraperitoneally with 0.2 ml of sterile, endotoxin free PBS, free MTP (100 ug/mouse), sham liposome, liposome encapsulated MTP (100 ug/mouse), or C. parvum (1.4 mg/mouse). Peritoneal macrophages from each treatment group (except C. parvum) were collected 48 after inoculation. C. parvum PEC were collected 4 days post inoculation. Washed PEC were adjusted to 5×10^6 cells/ml and incubated with 10^6 viable S. aureus organisms. Viable organisms remaining at selected time intervals were determined by plating on agar plates. The percentage of viable bacteria recorded at the indicated times represent the average obtained from three mice.

LOG RELATIVE FLUORESCENCE
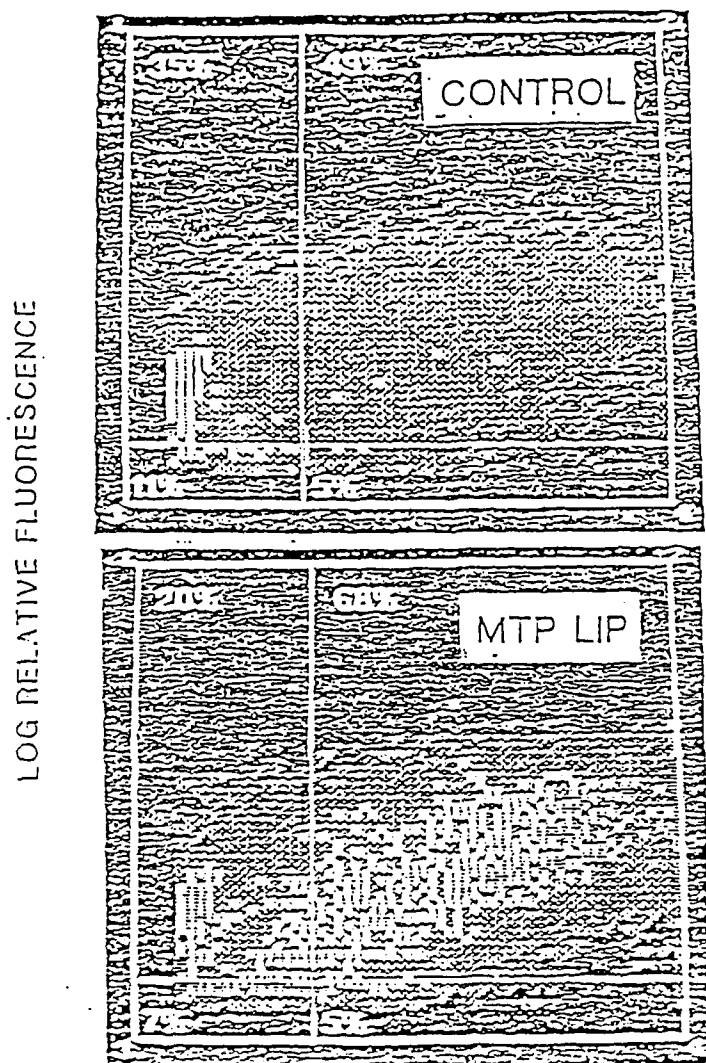
Figure 11. Cell Sorter Analysis of Liver
Macrophages Following Phagocytosis
of Fluorescent Latex Particles.

Mice were given intravenous injections
of liposome-encapsulated MTP-PE
(100μg/mouse) 48 hours prior to the
i.v. inoculation of fluorescent latex
particles. Livers were excised 18 hours
later and macrophages isolated and analyzed
in an EPICS IV Flow Cytometer. Movement to
right of the scale (bright dots) represents
increased numbers of fluorescent latex
particles phagocytosed by each cell.

FIGURES 12 & 13

A - TISSUE DISTRIBUTION OF C[14] LABELLED NON-ENCAPSULATED RIBAVIRIN
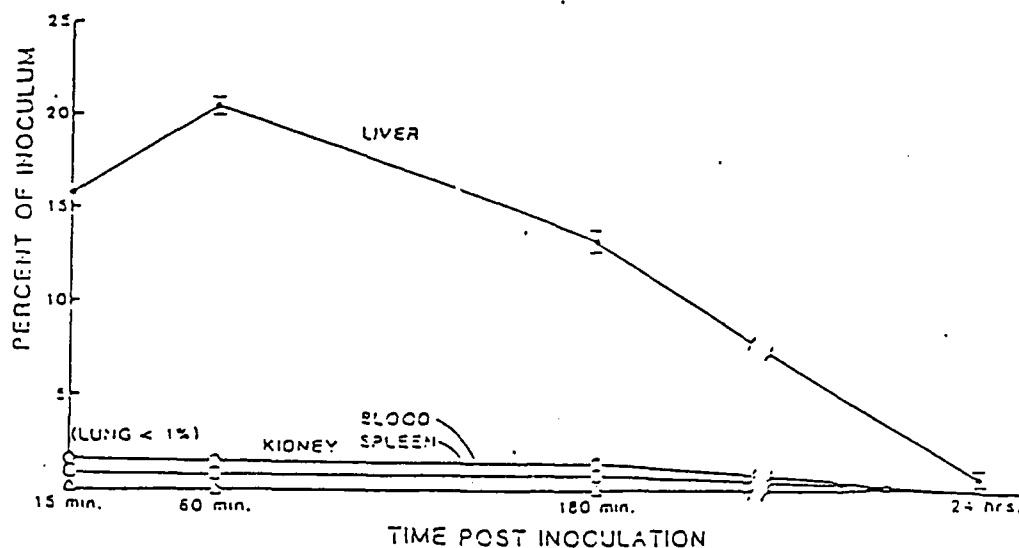
B - TISSUE DISTRIBUTION OF C[14] LABELLED LIPOSOME ENCAPSULATED RIBAVIRIN
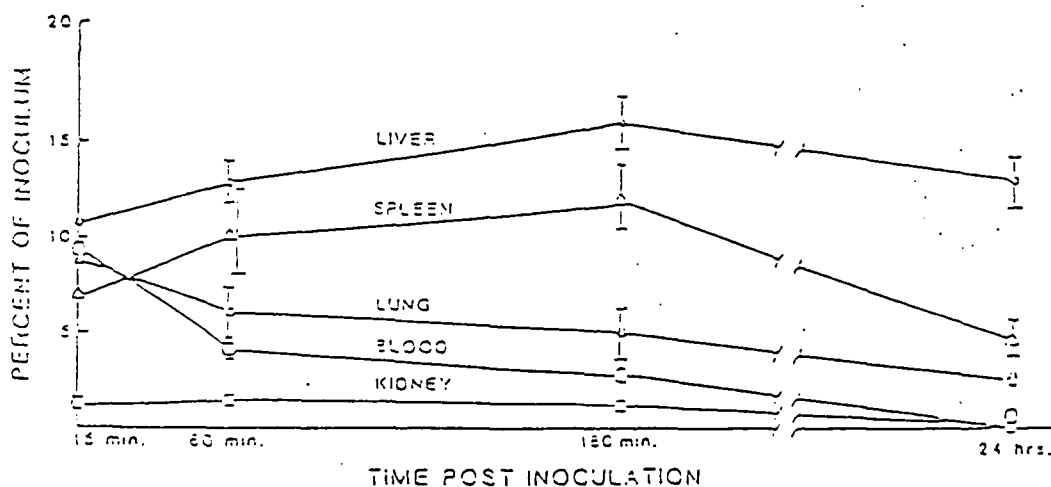
Figures 12 and 13. Tissue Distribution of C[14] Labeled Ribavirin

A. Non-encapsulated (free) ribavirin.

B. Liposome-encapsulated ribavirin. Four groups of 6,8 weeks old mice
    were inoculated intravenously with radiolabeled free or liposome-
    encapsulated ribavirin. Three mice from each time group were
    sacrificed by cervical dislocation and a blood sample taken prior
    to exsanguination. Livers, spleens, lungs and kidneys were removed,
    washed, and dried prior to weighing. Weighed samples were digested
    with tissue solubilizer and aliquots counted in a liquid scintillation
    counter.

FIGURE 14

RIBAVIRIN TREATMENT OF INFLUENZA VIRUS INFECTED MICE

Legend:
□ LIPOSOME ENCAPSULATED RIBAVIRIN DAY 0, DAY 2
▲ FREE (NONENCAPSULATED) RIBAVIRIN DAY 0, DAY 2
✕ SHAM LIPOSOME DAY 0
● FREE RIBAVIRIN DAY 0
○ RIBAVIRIN LIPOSOME DAY 0

Axes: PERCENT SURVIVAL (vertical) vs DAYS POST INFECTION (horizontal)
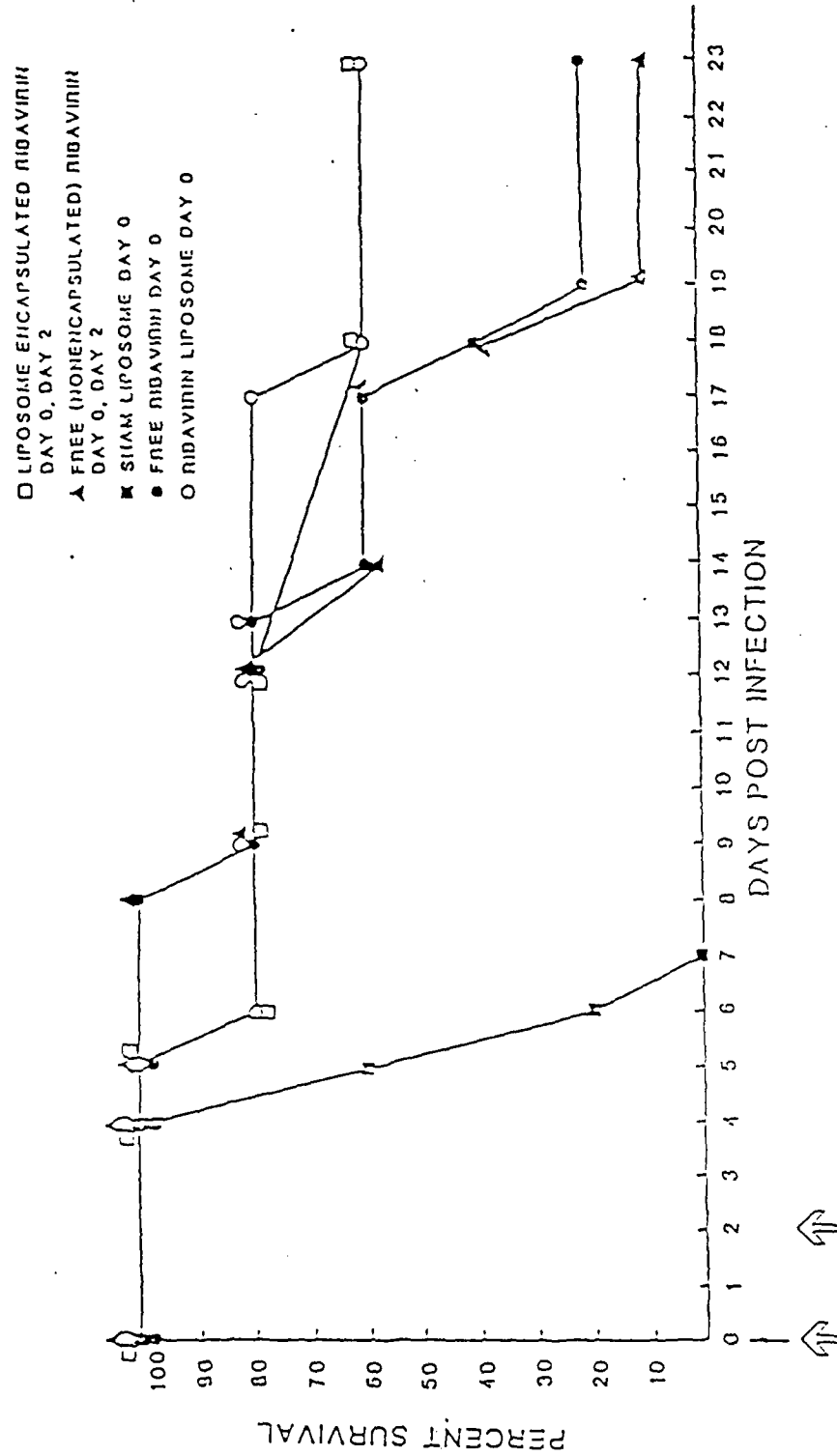
Figure 14 - Ribavirin Treatment of Influenza Virus Infected Mice. Mice were injected intranasally with $10 LD_{50}$ of influenza and treated intravenously with either free (10 mg/mouse) or liposome encapsulated (3 mg/mouse) ribavirin on the indicated days. Each group consisted of 10 mice. Arrows indicate inoculation days.

FIGURE 15

RIBAVIRIN TREATMENT OF HSV-1 PNEUMONITIS
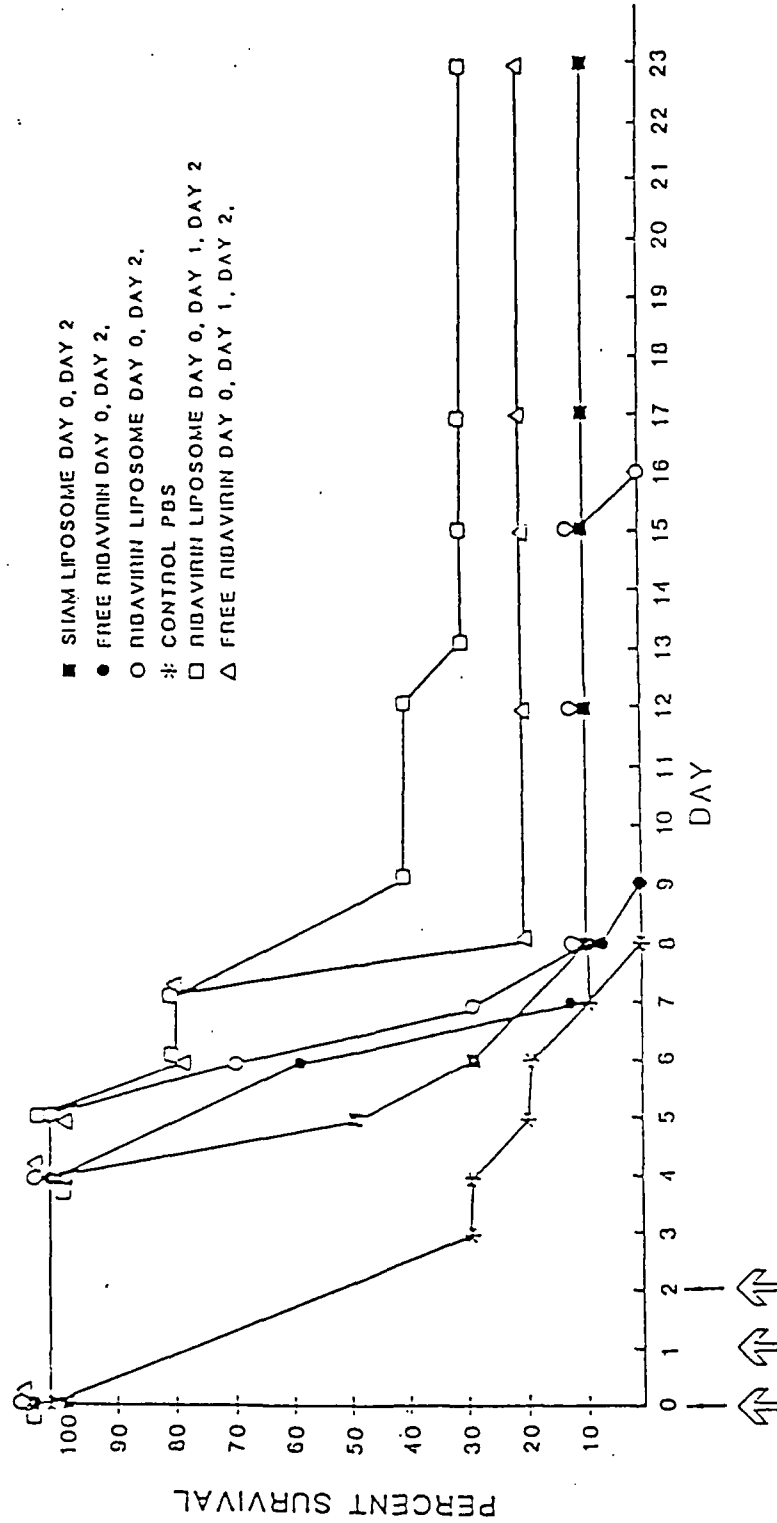
Figure 15 - Ribavirin Treatment of HSV-1 Pneumonitis. Mice were injected intranasally with 10LD50 of HSV-1 and treated with free (3 mg/mouse) or liposome encapsulated ribavirin (10 mg/mouse) on the days indicated. Each group consisted of 10 mice and were examined daily for 30 days. Arrows indicate inoculation days.
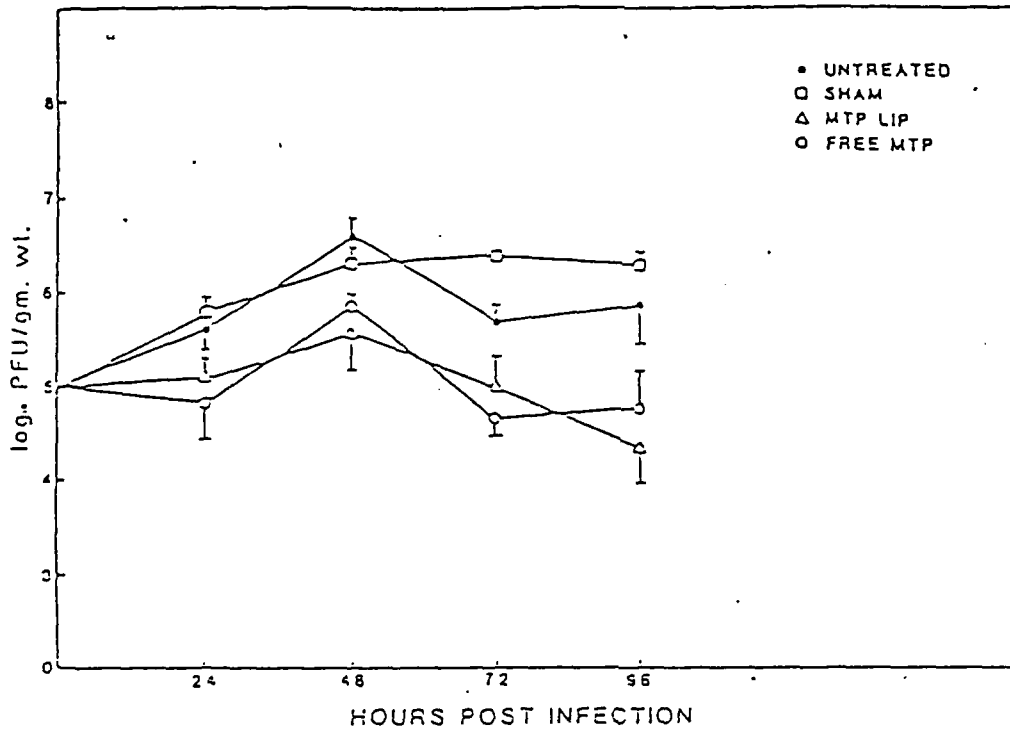
FIGURE 16

MTP TREATMENT OF HSV-1 PNEUMONITIS
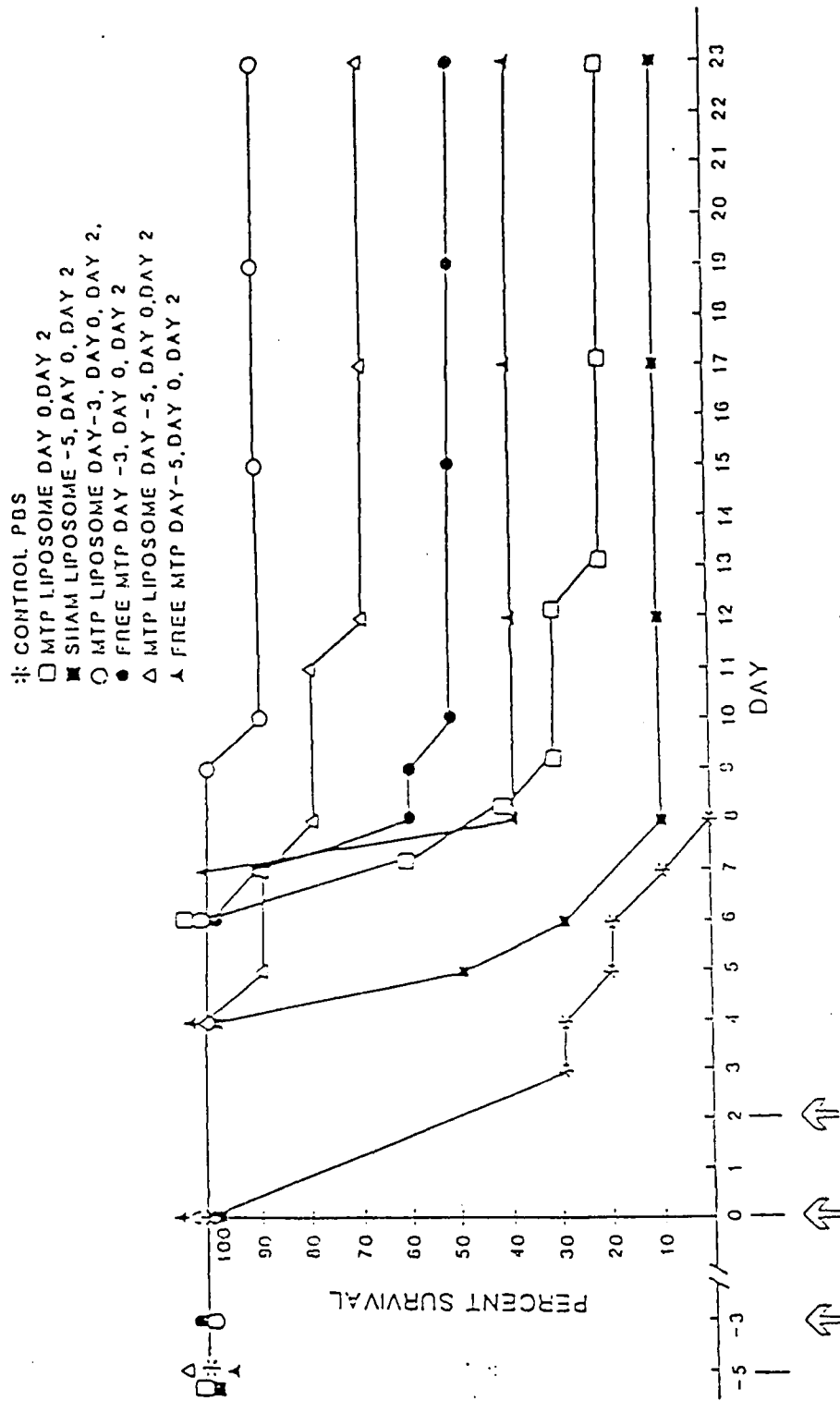
Figure 16 - MTP Treatment of HSV-1 Pneumonitis. Four-five week old mice were inoculated intravenously with 0.2 ml of either free or liposome encapsulated MTP ($100\mu g/dose$) at the times indicated and challenged intranasally with $10LD_{50}$ of HSV-1. Each group consisted of 10 mice and were examined daily for 30 days. Arrows indicate inoculation days.

FIGURES 17 & 18

HSV-1 TITRES IN LUNGS POST MTP TREATMENT

HSV-1 TITRES IN ADRENALS POST MTP TREATMENT
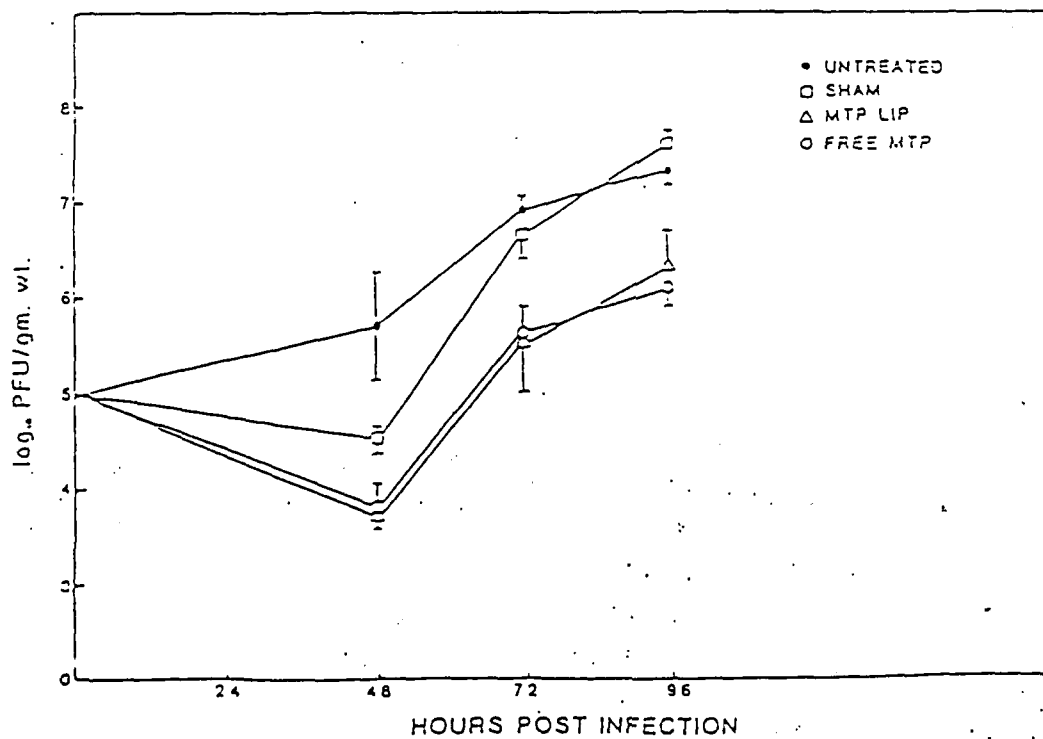
FIGURE 19
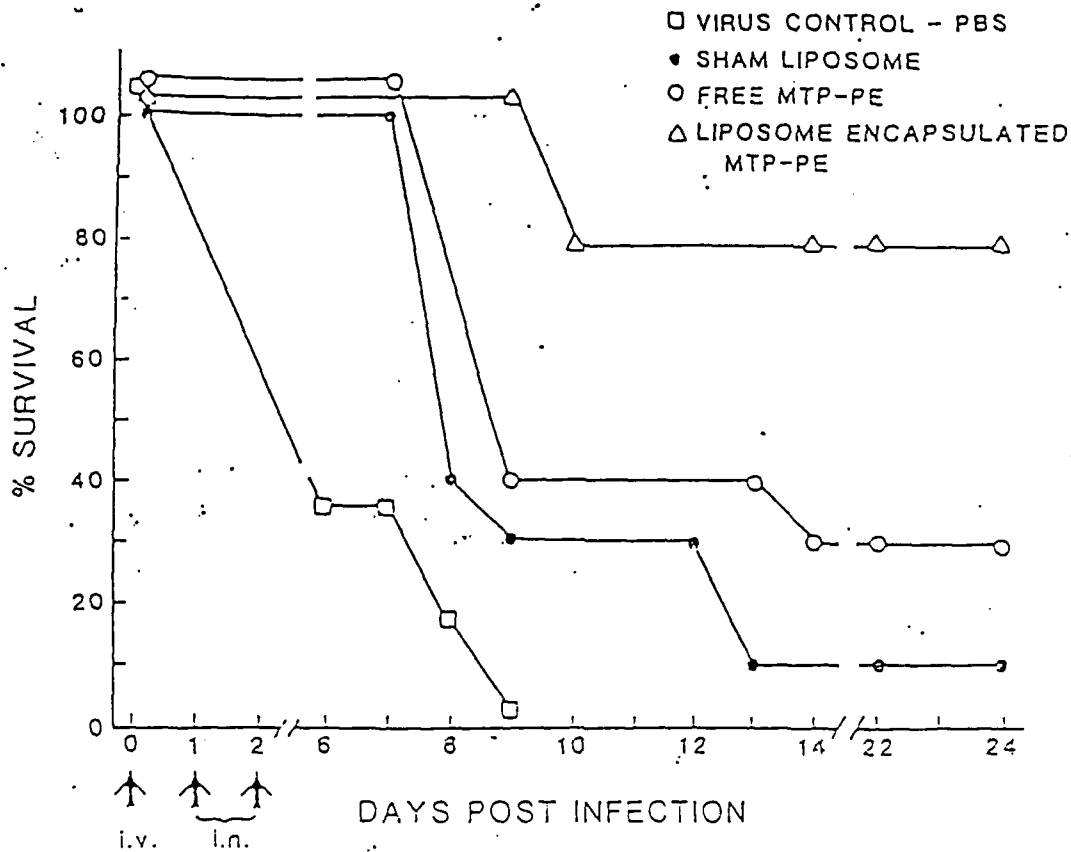
Figure 19. Therapeutic Activity of MTP-PE
in Treatment of HSV-1 Hepatitis.

Five-six week old mice were inoculated
intravenously with 10^4 p.f.u. of HSV-1
and treated on day 0 with 100µg (i.v.) of
either liposome-encapsulated or free MTP-PE.
This was followed by intranasal administration
of free MTP-PE (100µg) on days 1 and 2 post-
infection. Percent survival is based on 10
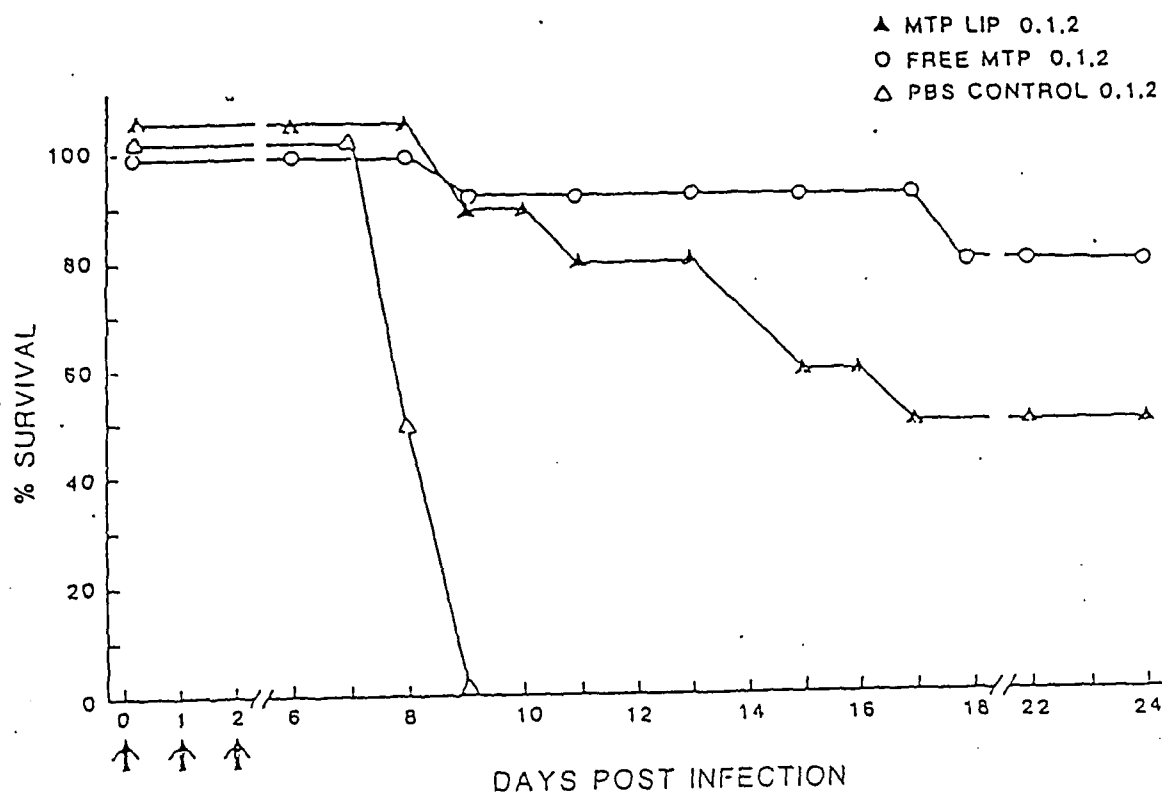mice per group. P<.05 for liposome-encapsulated
verses free MTP-PE

FIGURE 20

Figure 20. Intranasal MTP-PE Therapy of HSV-1 Encephalitis.

Four week old mice were given 5×10^5 p.f.u. of the MB strain of HSV-1 via footpad inoculation. Either free or liposome-encapsulated MTP-PE (100µg) was administered intranasally on days 0,1 and 2 post-infection. Percent survival was calculated from 10 mice per group. P<.05 for free verses liposome-encapuslated MTP-PE.
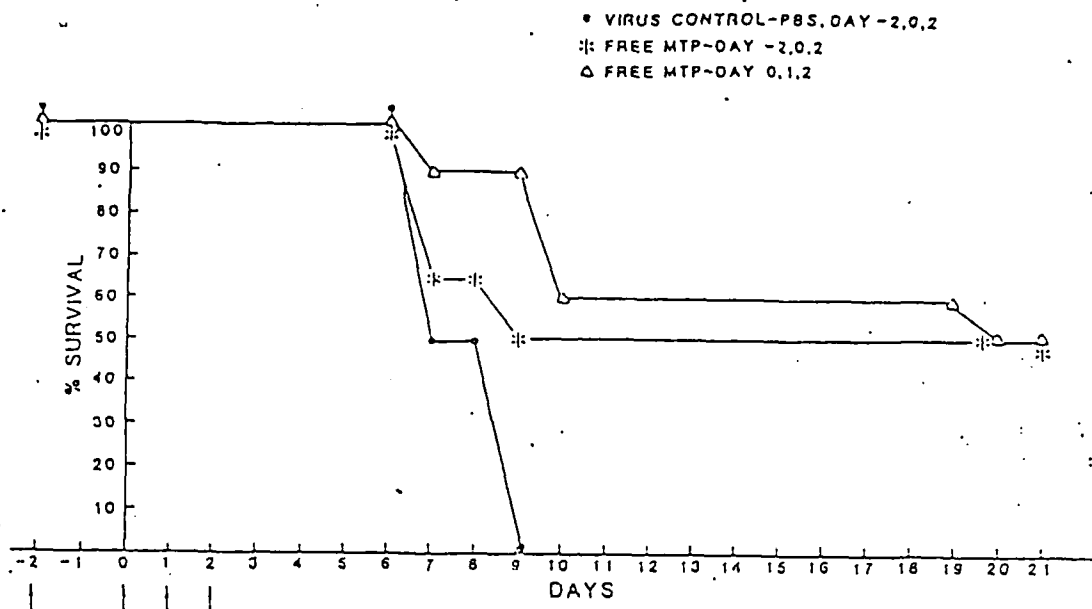
66

FIGURE 21

Figure 21.　　Intravenous MTP-PE Therapy of HSV-1
Encephalitis

Four week old mice were inoculated
as described in Figure 3a and
intravenously administered free MTP-PE
(100µg/mouse) on the days indicated.
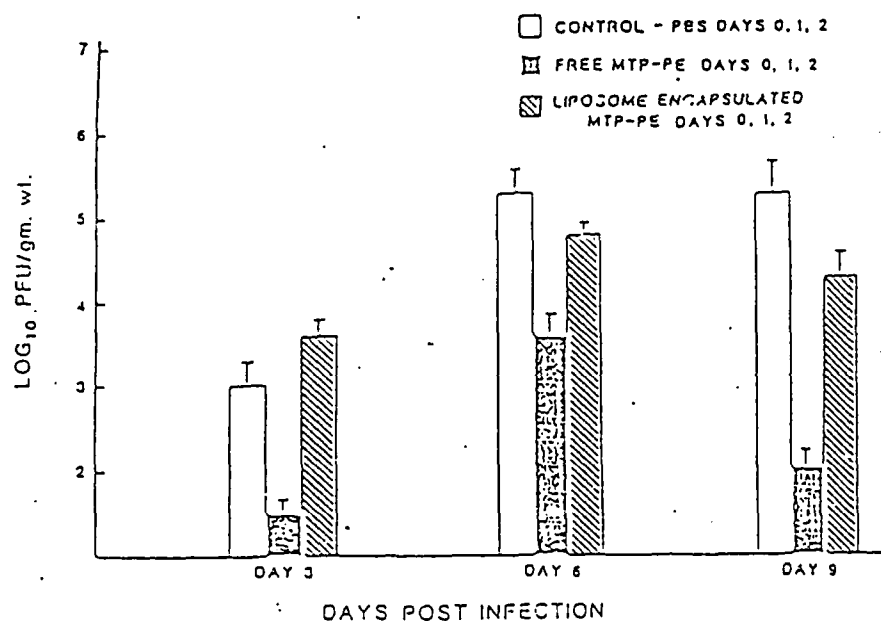Ten mice per group were analyzed.

FIGURE 22

Figure 22. Virus Titers in Spinal Cords of Mice
Receiving Intranasally Administered MTP-PE

Four week old mice were inoculated
and treated with MTP-PE (100μg/mouse)
as described in Figure 3a. Spinal cords were
removed and infectious virus determined by
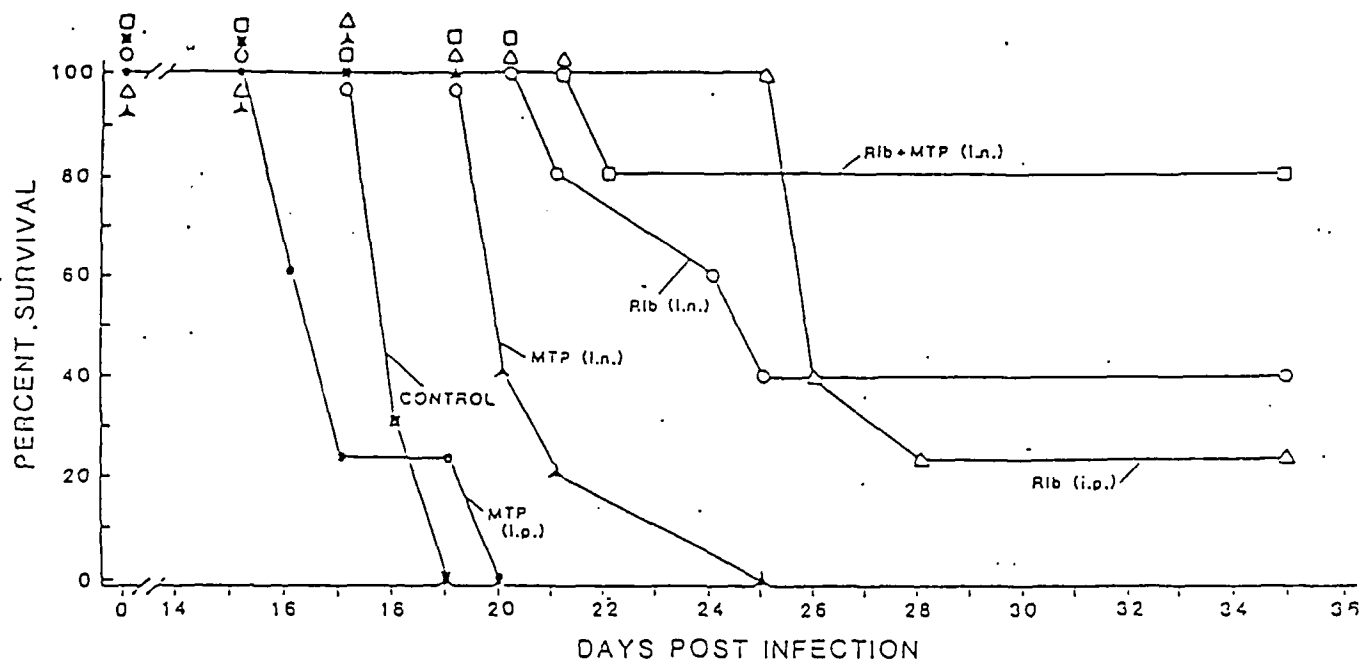plaquing on Vero cells.

FIGURE 23

Figure 23. Ribavirin and MTP-PE Therapy of
Pichinde Virus Infected Guinea Pigs

Strain 13 guinea pigs (female 300-350
grams) were inoculated intraperitoneally
with 1x10⁴ p.f.u. of Pichinde virus
(AN 4763 GP-pass 13) and treated with
ribavirin (15mg) and/or MTP-PE (200μg)
via intraperitoneally or intranasal
administration. See Tables 8 and 9
for complete description of drug dosage
and scheduling. Five guinea pigs per treatment
group were examined. P<.05 for combined i.n.
therapy (ribavirin + MTP) verses monotherapy
with either ribavirin or MTP.
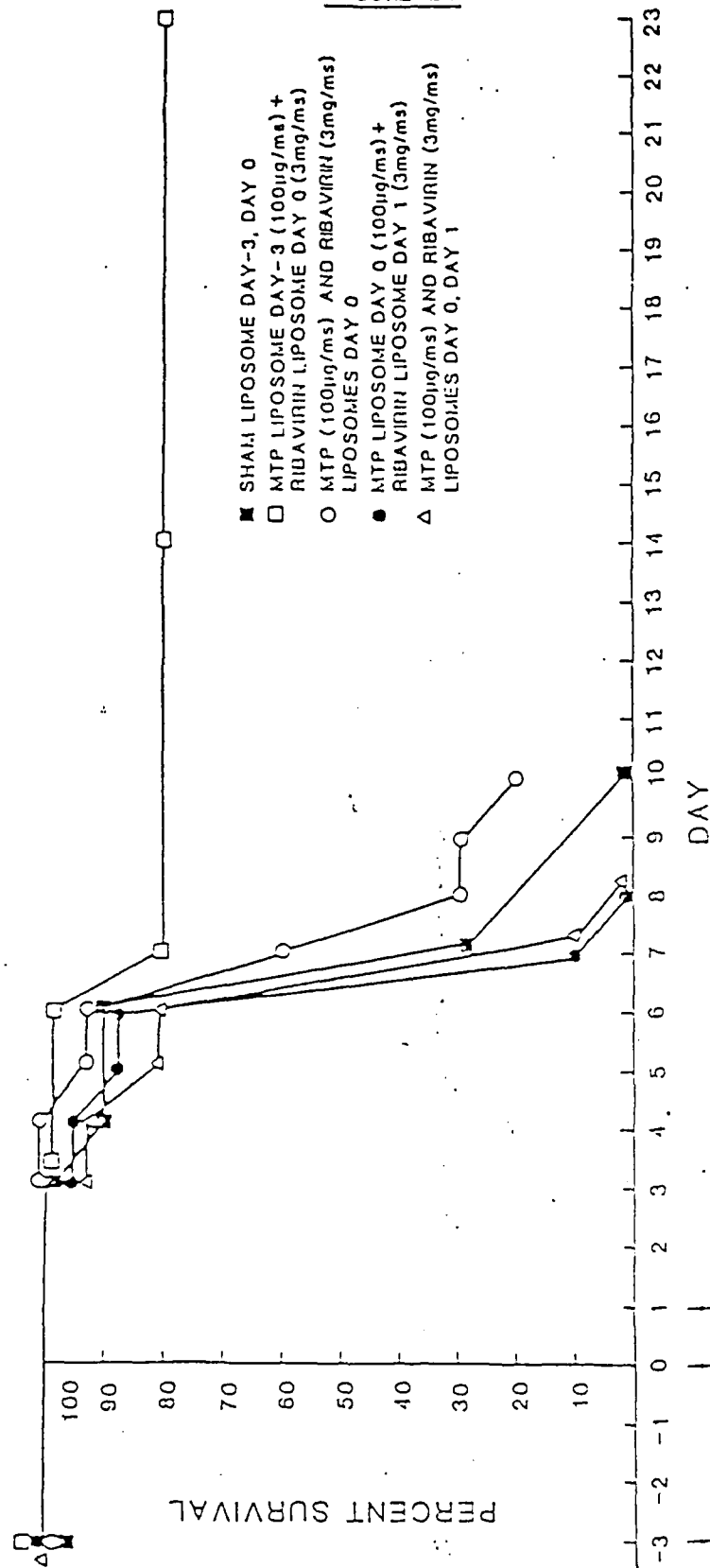
COMBINED MTP/RIBAVIRIN THERAPY OF HSV-I PNEUMONITIS.

FIGURE 24

PERCENT SURVIVAL

DAY

- ■ SHAM LIPOSOME DAY-3, DAY 0
- □ MTP LIPOSOME DAY-3 (100μg/ms) + RIBAVIRIN LIPOSOME DAY 0 (3mg/ms)
- ○ MTP (100μg/ms) AND RIBAVIRIN (3mg/ms) LIPOSOMES DAY 0
- ● MTP LIPOSOME DAY 0 (100μg/ms) + RIBAVIRIN LIPOSOME DAY 1 (3mg/ms)
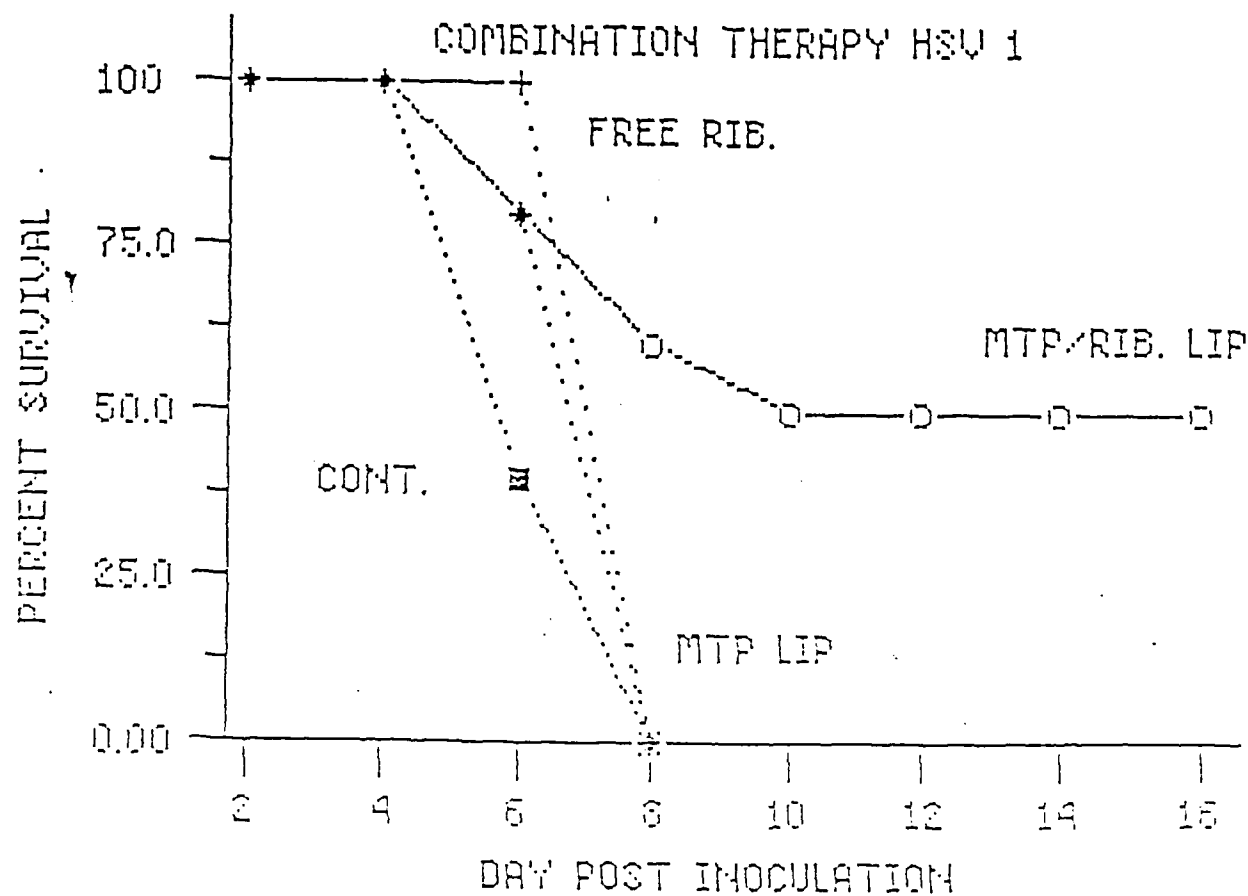- △ MTP (100μg/ms) AND RIBAVIRIN (3mg/ms) LIPOSOMES DAY 0, DAY 1

Figure 25. Combination therapy of HSV-1 pneumonitis. C₃H/Hen mice were
infected intranasally with 10 LD₅₀ of HSV-1 and then treated intravenously
with either free ribavirin (15 mg/mouse), liposome-encapsulated MTP-PE
(40 μg/mouse), or liposomes containing MTP-PE (40 μg) and ribavirin
(2.5 mg). This treatment was repeated two days following virus infection.